(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 August 2005 (04.08.2005)

PCT

(10) International Publication Number
WO 2005/070446 A1

(51) International Patent Classification[7]: A61K 38/00

(21) International Application Number:
PCT/US2005/001480

(22) International Filing Date: 18 January 2005 (18.01.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/537,221 15 January 2004 (15.01.2004) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** METHOD FOR TREATING CARDIAC REMODELING FOLLOWING MYOCARDIAL INJURY

(57) **Abstract:** The invention concerns methods for treating cardiac remodeling in a subject who has undergone myocardial injury, said method comprising the administration of natriuretic peptide to said subject. Preferably the natriuretic peptide is brain natriuretic peptide. The invention also concerns methods for treating structural heart disorders arising from myocardial injury, said method comprising the administration of a natriuretic peptide to a patient in need thereof.

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METHOD FOR TREATING CARDIAC REMODELING FOLLOWING MYOCARDIAL INJURY

This application claims priority to U.S. provisional application Serial No. 60/537,221. The 60/537,221 provisional application is herein incorporated by reference in its entirety.

Field of the Invention

The present invention concerns methods of treatment using one or more natriuretic peptides or derivatives thereof. More specifically, the invention concerns methods of treating or preventing cardiac dysfunction in a subject after said subject has undergone myocardial injury.

BACKGROUND

Myocardial infarction is a major cause of significant disability and death in the United States and in many other countries around the world, and accounts for approximately 2/3 of all heart failure. Hunt et al, AMERICAN COLLEGE OF CARDIOLOGY/AMERICAN Heart Association. ACC/AHA guidelines for the evaluation and management of chronic heart failure in the adult: executive summary. A report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to revise the 1995 Guidelines for the Evaluation and Management of Heart Failure). Journal of the American College of Cardiology 2001; 38: 2101-2113. Several disease-initiating events (e. g. myocardial infarction, untreated hypertension, congenital mutations of contractile proteins) can

result in a common heart disease phenotype that consists of dilation of the cardiac chambers, resulting in reduction in contractile function (i.e., a decrease in the fraction of total blood ejected from each chamber during systole) that leads to the clinical syndrome of heart failure. This phenotype generally involves a compensatory aspect that results from myocardial infarction when the normal compensatory hypertrophy of surviving, non-infarcted myocardium is insufficient. Often this compensatory mechanism is a result of the profibrotic response associated with cardiac injury.

Available therapies for heart dysfunction are insufficient, and new methods of treatment are needed. The heart responds to infarction by hypertrophy of surviving cardiac muscle in an attempt to maintain normal contraction. However, when the hypertrophy is insufficient to compensate, cardiac remodeling and reduced cardiac function result, leading to heart failure and death. Despite important advances in medical therapies for preventing cardiac dysfunction and heart failure after myocardial infarction, these problems remain a significant unsolved public health problem.

No pharmacological therapy for post MI cardiac remodeling is curative or satisfactory, and many patients die or, in selected cases, undergo heart transplantation. Presently available pharmacological therapies for reducing cardiac dysfunction and reducing mortality in patients with heart failure fall into three main categories: angiotensin-converting enzyme (ACE) inhibitors, beta adrenergic receptor (βAR) antagonists, and aldosterone antagonists. Despite reducing mortality, patients treated with these medicines remain at significantly increased rislc for death compared to age- matched control patients without heart failure. ACE inhibitors, ßAR antagonists and (at least one type of) aldosterone receptor antagonist can significantly reduce the

incidence and extent of cardiac dysfunction and heart failure after myocardial infarction.

ACE inhibitors are associated with cough in 10% of patients and can result in renal failure in the setting of bilateral renal artery stenosis or other severe kidney disease. βAR antagonists are associated with impotence and depression, and are contraindicated in patients with asthma; furthermore, patients may develop worsened heart failure, hypotension, bradycardia, heart block, and fatigue with initiation of βAR antagonists. Aldosterone receptor antagonism causes significant hyperkalemia and painful gynecomastia in 10% of male patients. Agents without a demonstrated mortality benefit are also associated with problems; most notable is the consistent finding that many cardiac stimulants improve symptoms, but actually increase mortality, likely by triggering lethal cardiac arrhythmias. In summary, presently available pharmacological therapies are ineffective and are limited by significant unwanted side effects, and so development of new therapies with improved efficacy and less severe side effects is an important public health goal.

SUMMARY OF THE INVENTION

The present invention is directed to the use of natriuretic peptides for the prevention and/or treatment of cardiac remodeling in a subject that has undergone myocardial injury. In a preferred embodiment, the natriuretic peptide(s) comprise brain natriuretic peptide (BNP), also known as nesiritide. In another embodiment, the invention is directed to the treatment of cardiac dysfunction, said treatment comprising the administration of a therapeutically effective amount of natriuretic peptide to a subject that has undergone myocardial injury.

In another related embodiment, the invention is directed to a method of alleviating or reversing the effect of TGFβ mediated cell activation in cardiac tissue on the expression of one or more genes associated with fibrosis, comprising contacting one or more cells or tissues in which the expression of said genes is altered as a result of TGFβ mediated activation, with BNP. In another related embodiment, the targeted gene(s) associated with fibrosis are selected from the group consisting essentially of Collagen1, Collagent 3, Fibronectin, CTGF, PAI-1, and TIMP3.

In another embodiment, the invention is directed to a method of inhibiting the production of Collagen 1, Collagen 3 or Fibronectin proteins by the administration of a therapeutically effective amount of BNP to a subject in need thereof.

In another related embodiment, the invention is directed to a method of inhibiting TGFβ mediated myofibroblast conversion by administration of a therapeutically effective amount of BNP to a mammalian subject in need thereof.

In another related embodiment, the invention is directed to a method of alleviating or reversing the effect of TGFβ mediated cell activation in cardiac tissue on the expression of one or more genes associated with cell proliferation, comprising contacting one or more cells or tissues in which the expression of said genes is altered as a result of TGFβ mediated activation, with BNP. In another related embodiment, the targeted gene(s) associated with cell proliferation are selected from the group consisting essentially of PDGFA, IGF1, FGF18, and IGFBP10.

In another related embodiment, the invention is directed to a method of alleviating or reversing the effect of TGFβ mediated cell activation in cardiac tissue on the expression of one or more genes associated with inflammation, comprising contacting one or more cells or tissues in which the expression of said genes is altered as a result of TGFβ mediated activation, with BNP. In another related embodiment,

the targeted gene(s) associated with inflammation are selected from the group comprise COX1, IL6, TNFα-inducted protein 6, TNF superfamily, member 4.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Gene expression changes induced by TGFβ and BNP in human cardiac fibroblasts at 24 and 48 h. Histograms show the number of gene expression changes that were up-regulated and down-regulated by TGFβ and BNP treatment. Hybridizations using fluorescently-labeled cDNA probes compare untreated (control) to TGFβ-treated cells and control to BNP-treated cells. See Experimental for details related to the gene expression values. Histogram bars: 24 h (white) and 48 h (black).

Figure 2. Effects of BNP on TGFβ-induced gene expression in human cardiac fibroblasts. Hybridizations using fluorescently-labeled cDNA probes compare TGFβ-treated to TGFβ BNP-treated cells at 24 and 48 h. Strong and weak effects represent 1.8- and 1.5- fold gene expression levels, respectively. See Experimental for details related to statistical significance. Histogram bars: no effect (white), weak effect (grey), and strong effect (black).

Figure 3. Gene expression patterns in TGFβ-treated human cardiac fibroblasts. Data was generated using the hierarchical clustering algorithm contained in Spotfire™ software. Each row represents one of 524 genes, and each column represents the results from duplicate hybridizations: (A) control vs. TGFβ, 24 h; (B) control vs. TGFβ, 48 h; (C) TGFβ vs. TGFβ + BNP 24 h; (D) TGFβ vs. TGFβ + BNP 48 h; (E) control vs. BNP 24 h; and (F) control vs. BNP 48 h. Normalized data values depicted in shades of red and green represent elevated and repressed expression, respectively. See Table 2 in Experimental section for gene identities and expression values.

Figure 4. Gene expression clusters in human cardiac fibroblasts: (A) fibrosis and ECM, (B) cell proliferation, and (C) inflammation. See Fig. 4 legend for descriptions of the hybridizations and gene expression color codes.

Figure 5. Effects of BNP on TGFβ-induced Collagen 1 (A and B) and Fibronectin (C and D) mRNA and protein levels in cultured human cardiac fibroblasts. Histograms show control cells (white), cells treated with BNP (gray), cells treated with TGFβ(black), and cells co-treated with BNP and TGFβ(hatched). (A and C) Real-time RT-PCR expression levels were normalized to 18S rRNA and plotted relative to the level in the 6 h control cells. Error bars reflect duplicate biological replicates; real-time RT-PCR reactions were performed in triplicate. (B and D) Western blot analyses are presented as mean ± SD from three separate experiments; *$p<0.01$ vs. control; **$p<0.01$ vs. TGFβ.

Figure 6. Effects of BNP on TGFβ–induced fibrotic and inflammatory genes. Real-time RT-PCR expression levels were normalized to 18S rRNA and plotted relative to the level in the 6 h control cells. See Fig. 5 for key to histogram bar labels and error bars.

Fig 7. Effect of PKG and MEK inhibitors on BNP-dependent inhibition of TGFβ signaling in human cardiac fibroblasts. (A) Western analysis of ERK phosphorylation. Cells were treated with BNP (0.5 μmol/L) in the presence or absence of KT5823 (1 μmol/L) or U0126 (10 μmol/L) for 15 min. (B) Western blot and (C) real-time RT-PCR analysis to detect Collagen 1 expression. Cells were treated with 5 ng/ml TGFβ and/or BNP (100 nmol/L, three times daily) in the presence or absence of KT5823 (1 μmol/L), U0126 (0.1-10 μmol/L) or PD98059 (10 μmol/L) for 48 h. Control (C); KT5823 (KT); U0126 (U); TGFβ (TGF).

Figure 8. Summary of BNP effects on gene expression in TGFβ–stimulated human cardiac fibroblasts.

Figure 9. Effects of BNP on TGFβ-stimulated fibroblast proliferation. Histograms show fold induction of BrdU labeled cells treated with TGFβ alone, BNP alone or co-treated with BNP and TGFβ. Cells were co-treated with BNP and TGFβ for 24 h, then labeled with BrdU and cultured for an additional 24 h. Pooled data represent the mean ± SD from three individual experiments: *$p < 0.01$ vs. the control; **$p<0.05$ vs. TGFβ.

Figure 10. Changes in plasma aldosterone level. The increased plasma aldosterone level by L-NAME/AngII was reduced by BNP ($p<0.05$, n=7)

Figure 11. Changes in heart/body weight ratio. BNP abolished L-NAME/AngII-induced increase in heart/body weight ratio ($p<0.01$, n=12)

Figure 12. Real time RT-PCR results. Expression of mRNA of collagen I (A), collagen III (B) and fibronectin (C) in the heart. BNP abolished the fibrotic genes that enhanced by L-NAME plus Angiotensin II ($p<0.01$ in all cases).

Figure 13. Cardiac function parameters including heart rate (A), stroke volume (B), ejection fraction (C), cardiac output (D), stroke work (E), maximum dP/dt (F), minimum dP/fy (G), and arterial elastance (H). L-NAME/AngII induced deterioration of cardiac function. Administration of BNP significantly improved cardiac function as judged by increases in stroke volume, ejection fraction, cardiac output, stroke work and decrease in arterial elastance ($p<0.001$, n=8). BNP also increased maximum dP/dt ($p<0.05$) and minimum dP/dt. BNP had no effect on heart rate.

DETAILED DESCRIPTION

A. Definitions

As used herein, any reference to "reversing the effect of TGF-β-mediated cell activation on the expression of a gene associated with fibrosis" means partial or complete reversal the effect of TGF-β-mediated cell activation of that gene, relative to a normal sample of the same cell or tissue type. It is emphasized that total reversal (i.e. total return to the normal expression level) is not required, although is advantageous, under this definition.

The term "cardiac remodeling" generally refers to the compensatory or pathological response following myocardial injury. Cardiac remodeling is viewed as a key determinant of the clinical outcome in heart disorders. It is characterized by a structural rearrangement of the cardiac chamber wall that involves cardiomyocyte hypertrophy, fibroblast proliferation, and increased deposition of extracellular matrix (ECM) proteins. Cardiac fibrosis is a major aspect of the pathology typically seen in the failing heart. The proliferation of interstitial fibroblasts and increased deposition of extracellular matrix components results in myocardial stiffness and diastolic dysfunction, which ultimately leads to heart failure. A number of neurohumoral or growth factors have been implicated in the development of cardiac fibrosis. These include angiotensin II (AII), endothelin-1 (ET-1), cardiotrophin-1 (CT-1), norepinephrine (NE), aldosterone, FGF2, PDGF, and transforming growth factor-β (TGFβ). TGFβ expression is also stimulated by AII and ET-1 in cardiac myocytes and fibroblasts, further supporting its involvement in cardiac fibrosis.

The term "cardiac dysfunction" refers to the pathological decline in cardiac performance following myocardial injury. Cardiac dysfunction may be manifested through one or more parameters or indicia including changes to stroke volume, ejection fraction,

end diastolic fraction, stroke work, arterial elastance, or an increase in heart weight to body weight ratio.

The terms "differentially expressed gene," "differential gene expression" and their synonyms, which are used interchangeably, refer to a gene whose expression is activated to a higher or lower level in a test sample relative to its expression in a normal or control sample. For the purpose of this invention, "differential gene expression" is considered to be present when there is at least an about 2.5-fold, preferably at least about 4-fold, more preferably at least about 6-fold, most preferably at least about 10-fold difference between the expression of a given gene in normal and test samples.

"Myocardial injury" means injury to the heart. It may arise from myocardial infarction, cardiac ischemia, cardiotoxic compounds and the like. Myocardial injury may be either an acute or nonacute injury in terms of clinical pathology. In any case it involves damage to cardiac tissue and typically results in a structural or compensatory response.

As used herein, "natriuretic peptides" means a composition that includes one or more of an Atrial natriuretic peptide (ANP), a Brain natriuretic peptide (BNP), or a C-type natriuretic peptide (CNP). It is contemplated that analogues and variants of these peptides be included in the definition. Examples of such include anaritide (ANP analogue of different length) or combinations of natriuretic peptide including but not limited to ANP/BNP, ANP/CNP, an BNP/CNP variants. Preferably, natriuretic peptide means BNP (nesiritide).

The terms "treating" or "alleviating" refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) the targeted pathologic condition or disorder. Those in need of treatment

include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented. In the treatment of a fibroproliferative disease, a therapeutic agent may directly decrease the pathology of the disease, or render the disease more susceptible to treatment by other therapeutic agents.

The term " subject" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, cats, cattle, horses, sheep, pigs, goats, rabbits, etc. Preferably, the subject is human.

Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

A "therapeutically effective amount", in reference to the treatment of cardiac or renal fibrosis, e.g. when inhibitors of the present invention are used, refers to an amount capable of invoking one or more of the following effects: (1) inhibition (i.e., reduction, slowing down or complete stopping) of the development or progression of fibrosis and/or sclerosis; (2) inhibition (i.e., reduction, slowing down or complete stopping) of consequences of or complications resulting from such fibrosis and/or sclerosis; and (3) relief, to some extent, of one or more symptoms associated with the fibrosis and/or sclerosis, or symptoms of consequences of or complications resulting from such fibrosis and/or sclerosis.

B. Modes of Carrying out the Invention

Natriuretic peptides comprise a family of vasoactive hormones that play important roles in the regulation of cardiovascular and renal homeostasis. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are predominantly produced in the heart and exert vasorelaxant, natriuretic, and anti-growth activities.

Binding of ANP and BNP to type-A natriuretic peptide receptor (NPRA) leads to the generation of cyclic guanosine monophosphate (cGMP), which mediates most biological effects of the peptides. Mice lacking NPRA exhibit cardiac hypertrophy, fibrosis, hypertension and increased expression of fibrotic genes including *TGFβ1*, *TGFβ3* and *Collagen 1*. Furthermore, targeted disruption of the BNP gene in mice results in cardiac fibrosis and enhanced fibrotic response to ventricular pressure overload, suggesting that BNP is involved in cardiac remodeling.

TGFβ mediates fibrosis by modulating fibroblast proliferation and ECM production, particularly of collagen and fibronectin. TGFβ also promotes the phenotypic transformation of fibroblasts into myofibroblasts characterized by expression of *α-smooth muscle actin*. Studies have demonstrated that increased myocardial TGFβ expression is associated with cardiac hypertrophy and fibrosis. Moreover, functional blockade of TGFβ prevents myocardial fibrosis and diastolic dysfunction in pressure overloaded rats, indicating that TGFβ has a crucial role in the process of myocardial remodeling, particularly in cardiac fibrosis. However, the implication of natriuretic peptide(s) in this process has not been previously explored.

The present invention is directed to the treatment or prevention of cardiac remodeling following myocardial injury. In a preferred embodiment, the myocardial injury comprises an acute myocardial infarction. Preferably the administration of natriuretic peptide occurs as soon as possible after the injury event.

In another embodiment, the invention involves the treatment of cardiac dysfunction in a subject in need thereof comprising the administration of a natriuretic peptide to a subject in need thereof wherein said administration occurs after said subject has undergone myocardial injury.

The manner of administration and formulation of the natriuretic peptide(s) useful in the invention will depend on the nature of the condition, the severity of the condition, the particular subject to be treated, and the judgment of the practitioner; formulation will depend on mode of administration. The peptides of the invention are conveniently administered by oral administration by compounding them with suitable pharmaceutical excipients so as to provide tablets, capsules, syrups, and the like. Suitable formulations for oral administration may also include minor components such as buffers, flavoring agents and the like. Typically, the amount of active ingredient in the formulations will be in the range of about 5%-95% of the total formulation, but wide variation is permitted depending on the carrier. Suitable carriers include sucrose, pectin, magnesium stearate, lactose, peanut oil, olive oil, water, and the like.

The peptides useful in the invention may also be administered through suppositories or other transmucosal vehicles. Typically, such formulations will include excipients that facilitate the passage of the compound through the mucosa such as pharmaceutically acceptable detergents.

The peptides may also be administered by injection, including intravenous, intramuscular, subcutaneous, intrarticular or intraperitoneal injection. Preferably the natriuretic peptide(s) are administered intravenously. Typical formulations for such use are liquid formulations in isotonic vehicles such as Hank's solution or Ringer's solution.

Alternative formulations include aerosol inhalants, nasal sprays, liposomal formulations, slow-release formulations, and the like, as are known in the art.

Any suitable formulation may be used. A compendium of art-known formulations is found in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Company, Easton, PA. Reference to this manual is routine in the art.

The dosages of the peptide(s) of the invention will depend on a number of factors which will vary from patient to patient. The dose regimen will vary, depending on the conditions being treated and the judgment of the practitioner. Further information regarding related formulations and dosages for brain natriuretic peptide can be found in the package insert or the latest version of Physicians Desk Reference (PDR) for nesiritide or the Natrecor® product.

It should be noted that the peptides useful for the invention can be administered as individual active ingredients, or as mixtures of several different compounds. In addition, the peptide(s) can be used as single therapeutic agents or in combination with other therapeutic agents. Drugs that could be usefully combined with these compounds include natural or synthetic corticosteroids, particularly prednisone and its derivatives, monoclonal antibodies targeting cells of the immune system or genes associated with the development or progression of fibrotic diseases, and small molecule inhibitors of cell division, protein synthesis, or mRNA transcription or translation, or inhibitors of immune cell differentiation or activation.

As implicated above, although the peptide(s) of the invention may be used in humans, they are also available for veterinary use in treating non-human mammalian subjects.

Further details of the invention will be apparent from the Experimental section as provided below.

EXPERIMENTAL

In vitro

Cell Culture

Two lots of primary human cardiac fibroblasts, derived from an 18-year old Caucasian male (lot 1) and a 56-year old Caucasian male (lot 2), were provided by Cambrex Bio Science (Walkersville, MD). Cells stained positive for α-smooth muscle actin and vimentin antibodies corroborating their identity as cardiac fibroblasts and myofibroblasts. Both lots were used for the real-time RT-PCR studies; lot 1 was used for the microarray analysis. Cells at passage 3-5 were cultured in FGM containing 15% FBS. At confluence, cells were split and cultured in 6-well plates for 24 h. Cells were changed to serum-free medium and treated with human BNP (American Peptide Company, Sunnyvale, CA) in the presence or absence of 5 ng/ml of TGFβ (R&D systems, Minneapolis, MN) for 6, 24 and 48 h. BNP and/or TGFβ–treated cells were also incubated in the presence of cGMP-dependent protein kinase (PKG) inhibitor KT5823 (1 μmol/L, Calbiochem, San Diego, CA), MAP kinase kinase (MEK) inhibitor U0126 (0.1-10 μmol/L, Sigma, St. Louis, MO) or PD98059 (10 μmol/L, Sigma) for 48 h. BNP (100 nmol/L) was added into the medium three times a day, such that the total calculated concentrations of exogenous BNP were 200 nmol/L, 600 nmol/L, and 900 nmol/L at 6, 24, and 48 h, respectively. This dosing protocol was necessary to maintain the levels of BNP in culture, since two distinct clearance pathways are responsible for the rapid degradation of natriuretic peptides. Without this treatment regime, it was found that BNP was significantly degraded in the cardiac fibroblasts; 50% of added BNP was metabolized within 24 h as measured by immunoreactive assays and cGMP stimulation cell bioassays.

Intracellular cGMP assay

Cells were cultured in 6-well plates for 24 h, then changed to serum-free medium, and pre-incubated with 0.1 mmol/L of 3-isobutyl-1-methylxanthine (IBMX) for 1 h before treating with 10^{-9} - 10^{-6} mol/L of BNP for 10 min. The medium was aspirated and 0.5 ml of cold PBS was added into each well. Cells were scraped and mixed with 2 volumes of cold ethanol by vortex. After a 5 min room temperature incubation, the precipitate was removed by centrifugation at 1500 x g for 10 min. The supernatant was dried by vacuum centrifugation, and levels of cGMP were measured using the cyclic GMP EIA kit (Cayman Chemical, Ann Arbor, MI).

BrdU incorporation

Cells were placed in 96-well plates and cultured for 24 h before changing to serum-free medium. Cells were treated with BNP (100 nmol/L, three times a day) in the presence or absence of 5 ng/ml of TGF-β for 24 h. Subsequently, 10 μmol/L of 5-bromo-2'-deoxyuridine (BrdU) was added to the cells, and they were cultured for an additional 24 h. BrdU incorporation was detected using the Cell Proliferation ELISA kit (Roche, Indianapolis, IN). Data was analyzed by ANOVA using the Newman-Keuls test to assess significance.

cDNA Microarray

Gene expression profiles were determined from cDNA microarrays containing 8,600 elements derived from clones isolated from normalized cDNA libraries or purchased from ResGen (Invitrogen Life Technologies, Carlsbad, CA). DNA for spotting was generated by PCR amplification using 5'amino-modified primers (BD Biosciences Clontech, Palo Alto, CA) derived from flanking vector sequences. Amplified DNA

was purified in a 96-well format using Qiagen's Qiaquick columns (Valencia, CA) according to the manufacturer's recommendations. Samples were eluted in Milli-Q purified water, dried to completion and resuspended in 7 μl of 3X SSC. A fluorescent assay using PicoGreen (Molecular Probes, Eugene, OR) was randomly performed on 12% of the PCR products to determine the average yield after purification; yields were ~1.5 μg of DNA which corresponds to a concentration of 214 μg/ml. Purified DNA was arrayed from 384-well microtiter plates onto lysine-coated glass slides using an OmniGrid II microarrayer (GeneMachines, San Carlos, CA). After printing, DNA was cross-linked to the glass with 65 mjoules UV irradiation and reactive amines were blocked by treatment with succinic anhydride.

mRNA Isolation, Labeling, and Hybridizations

Total RNA was extracted from cells using Qiagen's RNeasy kit; two wells from a 6 well plate were pooled to yield a total of 4 x 10^5 cells per treatment. RNA was amplified using a modified Eberwine protocol[51] that incorporated a polyA tail into the amplified RNA. Fluorescently-labeled cDNA probes were generated by reverse transcription of 4 μg of RNA with SuperScript II (Invitrogen Life Technologies, Carlsbad, CA) using anchored dT primers in the presence of Cy3 or Cy5 dUTP (Amersham, Piscataway, NJ). Labeled cDNA probe pairs were precipitated with ethanol and purified using Qiaquick columns. Twenty μg each of poly(A) DNA, yeast tRNA, and human Cot1 DNA (Applied Genetics, Melbourne, FL) was added to the eluant. The samples were dried to completion and resuspended in 12.5 μl 3XSSC, 0.1%SDS. Probes were heated to 95°C for 5 minutes, applied to the arrays under a 22 mm^2 cover slip and allowed to hybridize for at least 16 h at 65°C. The arrays were

washed at 55°C for 10 minutes in 2XSSC, 0.1% SDS, followed by two washes at room temperature in 1XSSC (10 min) and 0.2XSSC (15 min). Hybridization of each fluorophore was quantified using an Axon GenePix 4000A scanner.

Microarray Data Analysis

Differential expression values were expressed as the ratio of the median of background-subtracted fluorescent intensity of the experimental RNA to the median of background-subtracted fluorescent intensity of the control RNA. For ratios greater than or equal to 1.0, the ratio was expressed as a positive value. For ratios less than 1.0, the ratio was expressed as the negative reciprocal (i.e., a ratio of 0.5 = -2.0). Median ratios were normalized to 1.0 using two pools of 3000 randomly chosen cDNAs in each pool. Six replicates of each of the two pools were printed in 4 evenly distributed blocks of the array. Expression data was rejected if neither channel produced a signal of at least 2.0-fold over background. Differential expression ratios were determined as the mean of the two values from dye-swapped duplicates.

A statistically significant differential expression threshold value was empirically determined according to the method of Yang *et al.*[53] Seven independent self-self- hybridizations were performed in which the same RNA sample was labeled with Cy3 dUTP and Cy5 dUTP and hybridized to arrays containing 8,448 elements. Only elements that gave a signal greater than 2.0-fold over background in at least one of the dyes were considered in the analysis. Expression ratios were converted to $\log_{(2)}$ and normalized to a mean = 0. Combining data from all hybridizations, the 3 standard deviation limit was equivalent to a 1.48 fold change (+/- 0.563 $\log_{(2)}$). Of the 45,633 elements analyzed, 0.85% fell outside this threshold. Therefore, at this standard deviation limit, genes with fold changes greater than 1.48 can be considered

differentially expressed at a 99% confidence level for any given hybridization. The percentage of elements that reproducibly fell outside the 3 standard deviation limit between any two duplicates of the seven self-self hybridizations was determined by comparing all 21 pair-wise combinations. An average of 18.9 elements +/- 15.6 per hybridization duplicated at a fold change of 1.5, corresponding to a false positive rate of 0.29%. At a fold change of 1.8, an average of 0.71 elements +/-0.97 duplicated, corresponding to a false positive rate of 0.01%. A 1.8-fold threshold value was used to identify differentially expressed genes, except in Fig. 3, a 1.5-fold threshold value was used to designate "weak effects".

Real-time RT-PCR

Real-time RT-PCR[18] was performed in a two-step manner. cDNA synthesis and real-time detection were carried out in a PTC-100™ Thermal Cycler (MJ Research Inc, Waltham, MA) and an ABI Prism™ 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), respectively. Random hexamers (Qiagen, Valencia, CA) were used to generate cDNA from 200ng RNA as described in Applied Biosystems User Bulletin #2. TaqMan™ PCR Core Reagent Kit or TaqMan™ Universal PCR Master Mix (Applied Biosystems) were used in subsequent PCR reactions according to the manufacturer's protocols. Relative quantitation of gene expression was performed using the relative standard curve method. All real-time RT-PCR reactions were performed in triplicate.

Sequence specific primers and probes were designed using Primer Express Version 2 software (Applied Biosystems). Sequences of primers and probes can be found in Table 1 below. Expression levels were normalized to 18S rRNA. The

selection of 18S rRNA as an endogenous control was based on an evaluation of the ΔC_T levels (Applied Biosystems document # 4308134C) of 6 "housekeeping" genes: *Cyclophilin A*, *18S*, *GAPDH*, *β-actin*, *β-Glucuronidase*, and *Hypoxanthine Guanine Phosphoribosyl Transferase*. The ΔC_T levels of *18S* did not differ significantly between treatment conditions; thus, they were expressed at constant levels between samples.

Table 1. Real-time PCR primers and probes.

Western blot analysis

Gene	Forward	Probe	Reverse
18S	5-GCGCCTAGAGGTGAAATTCTTG-3'	5'-6FAM-ACCGCGCCGCAAGACGGACCAG-TAMRA-3'	5'-CATTCTTGGCAAATGCTTTCG-3'
Collagen1	5'-CGAATTCGGCTTCGACGTT-3'	5'-6FAM-TCTGCTTCCTGTAAACTCCCTCCATCCG-TAMRA-3'	5'-TTCAGTTTGGGTTGCTTGTCTGT-3'
Fibronectin	5'-AGATCTACCTGTACACCTTGAATGACA-3'	5'-6FAM-TGTGGTCATCGACCGCCTCCA-TAMRA-3'	5'-CATGATACCACCAAGGAATTGG-3'
TIMP3	5'-TGTGTCATGTGTAGGCTGTAATATGTG3'	5'-6FAM-CACATCCCGCCATTTTCCTGAATCAA-TAMRA-3'	5'-GCCTAGAAGTATTTTCCTCTCCATTC-3'
PAI-1	5'-GCCTGACTTCACGAGTCTTTCA-3	5'-6FAM-ACCAAGACCCTCTCCACGTCCCG-TAMRA-3'	5'-GTTCACCTCGATCTTCACTTTCTG-3'
CTGF	5'-TGTGTGACGAGCCCAAGGA-3'	5'-6FAM-CTCCCCTCCCGGCTTACCGA-TAMRA-3'	5'-TAGTTCGGTCTCGCCCAAAC-3'
IL11	5'-AGAACACCGAATTAAATGTGTCATACA-3'	5'-6FAM-AGACAAATCCGCCCTCAAGTCGA-TAMRA-3'	5'-CCCAGTTACCCAAGCATCCA-3'
COX2	5'-GCTCAAACATGATGTTTCCATTC-3'	5'-6FAM-TTGCCCAGCACTTCACCCATCAG-TAMRA-3'	5'-GCCCTCGCTTATGATCTGTCTT-3'
IL6	5'-ATGTACCATCCGCACCTCAGAT-3'	5'-6FAM-TCGTCAGAAACCTGTCCACTCCGCA-TAMRA-3'	5'-TAACGCTCATACTTTAGTTCTCCATAGA-3'
α-smoothmuscle actin	5'-GCCCAGAGACCCTGTTCCA-3'	5'-6FAM-CCCAGCAGACTCCATCCCGA-TAMRA-3'	5'-TGATGCTGTTGTAGGTGGTTTCA-3'

Cells were cultured in 6-well plates and treated with BNP (100 nM, three times daily) in the presence or absence of 5 ng/ml TGFβ for 48 h. Lysis was induced with 0.2 ml of buffer containing 20 mM Tris-HCL, pH 7.9, 137 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM NaF, 1mM β-glycerophosphate, and protease inhibitor cocktail. The protein concentration of each lysate was measured using coomassie protein reagent from PIERCE. Twenty μg of protein from each sample was loaded and electrophoresed on 4-12% gradient polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes (Invitrogen, San Diego, CA). The membranes were incubated with rabbit anti-human Collagen 1 antibody (Cortex Biochem, San

Leandro, CA), HRP-conjugated anti-human Fibronectin antibody, or goat anti-Actin antibody (Santa Cruz Biotehnology, Santa Cruz, CA) in TBST buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20, and 5% nonfat dried milk at 4°C for ~16 h. For ERK phosphorylation, cells were treated with 0.5 μmol/L BNP in the presence of 1 μmol/L KT5823 or 10 μmol/L U0126 for 15 min; the membranes were incubated with rabbit anti-human phospho-ERK 1/2 antibody or rabbit anti-human ERK 1/2 antibody (Cell Signaling, Beverly, MA). For secondary antibody detection, membranes were incubated with HRP-conjugated anti-rabbit antibody or anti-goat antibody at room temperature for 1 h and washed 3 times with TBST buffer. The blots were soaked in ECL Plus reagent for 5 min and exposed to KODAK x-ray film. Signals were identified and quantified using a Typhoon Scanner and Densitometer from Amersham Biosciences (Piscataway, NJ). Data was analyzed by ANOVA using the Newman-Keuls test to assess significance.

Results

cGMP Production in Cardiac Fibroblasts

To determine if NPRA was expressed in the cultured fibroblast cells, cGMP accumulation assays were utilized. BNP dose-dependently induced intracellular cyclic GMP production in cardiac fibroblasts with an EC50 of 50 nmol/L. These results are consistent with the report of Cao and Gardner showing NPRA expression in cardiac fibroblasts.

Effects of BNP on TGFβ–Induced Fibroblast Proliferation

To examine the effects of TGFβ and BNP on cell proliferation, BrdU incorporation was measured in cardiac fibroblasts treated with TGFβ in the presence or absence of

BNP. TGFβ modestly increased (~50%) cardiac fibroblast proliferation, and BNP inhibited TGFβ–induced proliferation by ~65% (Figure 9).

Effects of BNP on TGFβ-Induced Gene Expression

In order to determine the effects of BNP on gene expression profiles induced by TGFβ in cardiac fibroblasts, a microarray analysis was performed. Fluorescently-labeled cDNA probes were prepared from pooled mRNAs generated from duplicate wells of cells from four groups: unstimulated (control), TGFβ-treated, BNP-treated, and co-treated with TGFβ and BNP for 24 and 48 h (as described above). Arrays were probed in duplicate for a total of 12 hybridizations (6 at each time point): control compared to TGFβ-treated, TGFβ-treated compared to TGFβ + BNP-treated, and control compared to BNP-treated.

It was observed that BNP had no significant effects on gene expression in unstimulated human cardiac fibroblasts (Fig 1). In contrast, TGFβ induced 394 and 501 gene expression changes at 24 and 48 h, respectively. These differentially expressed genes represent ~7-8% of the target genes on the array. Interestingly, BNP had dramatic effects on the gene expression changes induced by TGFβ (Fig 2). Approximately, 88% and 85% of TGFβ–regulated gene expression events were opposed by BNP at 24 and 48 h, respectively. These results demonstrate that BNP has strikingly different effects on gene expression in TGFβ stimulated fibroblasts compared to unstimulated cells.

Gene Expression Clustering

To identify different gene expression patterns following TGFβ stimulation, we performed a hierarchical cluster analysis. A visualization of this analysis is shown in

Fig. 3. A complete listing of differentially expressed genes is provided in Table 2. The clustered expression patterns showed temporal effects of TGFβ responsive genes (compare A to B). In addition, the dramatic effects of BNP in opposing TGFβ induced up- and down-regulated gene changes were revealed in the clusters (compare A and B to C and D). The insignificant effects of BNP on gene expression in unstimulated cardiac fibroblast cells were evident in groups E and F.

Genes were grouped according to functional categories by using a combination of gene expression clustering and functional annotations. A cluster of genes involved in fibrosis and ECM production was up-regulated in cells stimulated with TGFβ; these genes were down-regulated when treated with BNP (Fig. 4a). This cluster includes extracellular matrix components: *Collagen 1a2* (*COL1A2*), *Collagen 15A* (*COL15A*), *Collagen 7A1* (*COL7A1*), *Microfibril-associated glycoprotein-2 (MAGP2), Matrilin 3 (MATN3), Fibrillin 1 (FBN1),* and *Cartilage oligomeric matrix protein (COMP).* Also included in the cluster are known markers of fibrosis such as *TIMP3, CTGF, IL11, and SERPINE1 (PAI-1).* Furthermore, the cluster revealed that BNP opposed TGFβ-induction of myofibroblast markers including *α-smooth muscle actin 2 (ACTA2)* and *non-muscle myosin heavy chain (MYH9).*

Many genes involved in cell proliferation were also regulated by TGFβ and were opposed by BNP (Fig. 4B). For example, TGFβ induced the expression of positive regulators of cell proliferation, including *PDGFA, IGFBP10, IGF1, and Parathyroid hormone-like hormone (PTHLH).* It was also found that TGFβ down-regulated both positive and negative regulators of proliferation, such as, *CDC25B* and *Cullin 5 (CUL5),* respectively. All of these TGFβ–regulated gene events were opposed by BNP.

BNP affected TGFβ–induced genes involved in inflammation (Fig. 4C). For example, BNP reversed TGFβ-induction of *PTGS2 (COX2), TNF α-induced protein 6 (TNFAIP6),* and *TNF superfamily, member 4 (TNFSF4)* (Fig 4C and data not shown). *TNFAIP6* and *TNFSF4* were not included in Fig 4C, since some of the data points at 48 h did not meet acceptable criteria (see Experimental); at 24 h both genes were elevated ~3–fold by TGFβ and opposed by BNP. TGFβ also down-regulated many pro-inflammatory genes including *IL1B, CCR2 (MCP1-R)*, CXCL1 (GRO1), CXCL3 (GRO3), and CCL13 (MCP4), which were reversed by BNP. The significance of these inflammatory changes is discussed below.

Table 2. Expression data for differentially expressed genes in TGFβ-treated human cardiac fibroblasts. Median differential expression values are shown for each hybridization: control vs. TGFβ 24 h (column 2); control vs. TGFβ 48 h (column 3); TGFβ vs. TGFβ + BNP 24 h (column 4); TGFβ vs. TGFβ + BNP 48 h (column 5); control vs. BNP 24 h (column 6); and control vs. BNP 48 h (column 7).

Clone ID	TGF 24 h	TGF BNP 24 h	BNP 24 h	TGF 48 h	TGF BNP 48 h	BNP 48 h	Symbol	Name	Accession
P00777_A03	2.5	-2.8	1.1	1.5	-1.6	1.1		EST	
P00777_A04	8.9	-5.7	1.2	3.3	-2.4	1		EST	
P00777_A12	2.1	-2.4	-1	1.8	-1.9	-1.1		EST	
P01061_E01	2.7	-3	1	2.6	-2.8	-1		EST	
P01061_B10	-2.7	2.3	1.1	-4	2.4	-1.2		EST	
P01077_A08	-1.8	3.1	1.3	-2.2	1.9	1.2		No Sequence	
P01111_A08	-1.3	1.4	1.3	-1.8	1.7	1.1		EST	
P01113_E11	-1.7	1.8	1.1	-1.8	1.6	-1		EST	
P01111_F07	-4.5	5.5	1.3	-5.3	4.2	1.1		EST	
P01111_A07	2	-2.7	1.3	1.4	-1.5	-1.1		EST	
P01110_G03	-1.2	1.5	1.3	-3.9	2.1	1.1		No Sequence	
P01108_G07	4.2	-4.4	-1.1	3.9	-4.5	-1		EST	
P01099_G03	-1.9	1.9	1.1	-2.2	1.9	1.2		EST	
P01113_B03	6.4	-5.1	1	4.3	-3.7	-1		EST	
P01080_A11	4	-3	1	4.2	-4.1	-1		EST	
P01076_E01	-1.7	1.8	1.1	-1.8	1.8	-1.1		EST	
P01075_H09	-3.1	3.6	1.4	-2.9	3.2	1.4		No Sequence	
P01139_D10	3	-2.6	1.1	2.1	-2.1	1		EST	
P01132_B01	-2.1	2	1	-1.4	1.3	1		EST	
P01123_H03	2.2	-2.2	1.2	1.9	-1.9	1.1		EST	
P01117_D08	-1.7	1.5	1.1	-4.9	2.4	-1		EST	
P01115_F08	-2.2	1.6	-1	-2.3	1.7	-1		EST	
P01081_F02	2.4	-1.8	1.2	2.4	-2.1	1.1		No Sequence	
P01087_A12	2.4	-2	1	2.6	-2.6	-1		EST	
P01077_A02	2.2	-2	1	1.4	-1.3	-1		No Sequence	

P01136_G11	-2	2.5	1.3	-3	2.5	1		EST	
P01130_B03	-3.3	3.5	1.1	-4.2	5.3	1.1		EST	
P01124_A05	-1.2	-1	1.1	-1.8	1.5	1		EST	
P01124_A10	2.1	-2	-1	2.7	-2.5	-1.1		EST	
P01124_B04	-1.9	2	1.3	-1.6	1.7	1.1		EST	
P01120_G06	-2.3	2.2	-1.1	-2.4	2.2	-1.1		EST	
P01117_B11	1.8	-2.4	1	2.4	-2	1		EST	
P01116_A02	-3.1	2.7	1.1	-3.7	2.2	-1.4		EST	
P01088_C10	2.1	-2	-1	1.6	-2.1	-1.1		EST	
P01093_C04	2.6	-2.3	1	1.8	-1.9	-1		EST	
P01095_H01	-1.8	1.8	1	-1.4	1.2	1		EST	
P01099_D03	1.9	-1.8	1.1	1.1	-1.2	1.1		EST	
P01100_A07	1	-1	1.1	-3	1.7	-1.1		EST	
P01100_D09	-1.6	1.6	-1	-2.1	1.8	-1.1		EST	
P01101_C11	-2.4	1.7	-1	-1.4	1.6	1		No Sequence	
P01101_E11	-1.4	1.5	1.1	-2	1.8	-1		EST	
P01103_H04	-3.2	2.9	1.1	-5.6	4.3	-1		EST	
P01104_A09	-1.9	1.6	1.1	-1.8	1.5	1		No Sequence	
P01104_E03	-2.5	2.3	-1	-2.8	2	-1.1		EST	
P01104_G04	2.5	-2	-1	1.1	-1.3	-1.1		EST	
P01104_G12	-3.7	2.7	-1.1	-4.9	3.2	-1		EST	
P01105_A05	2.3	-2.3	1.3	1.3	-1.3	1		EST	
P01105_D09	1.8	-1.1	1.1	1.8	-2.1	-1		EST	
P01109_A01	-1.4	1.4	1.2	-2.2	1.7	1.1	A2M	alpha-2-macroglobulin	NM_000014
P01109_G11	1.4	-1	1.1	2	-1.6	1	ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	NM_004915
P01092_E08	2.3	-2	1.2	1.5	-1.3	1.1	ACLY	ATP citrate lyase	NM_001096
P01088_C02	-1.9	1.8	1.2	-2.1	2	1	ACO1	aconitase 1, soluble	NM_002197
P00777_G09	2.6	-2.2	-1.5	1	1	-1.3	ACTA1	actin, alpha 1, skeletal muscle	NM_001100
P01094_F04	2.6	-2.5	-1.4	-1	-1	-1.4	ACTA2	actin, alpha 2, smooth muscle, aorta	NM_001613
P01091_G04	1.9	-1.6	1.1	1.2	-1.3	-1	ACTR3	ARP3 actin-related protein 3 homolog (yeast)	NM_005721
P01096_D02	-1.3	1.5	1.1	-2.3	2.2	1	ADAMTS1	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	NM_006988
P01097_D04	1.7	-1.9	-1	2.1	-1.8	-1.1	ADAMTS6	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 6	NM_014273
P01092_D03	-6.5	6	-1.1	-6.3	6.5	-1	ADFP	adipose differentiation-related protein	NM_001122
P01070_D09	-5	4.1	1.3	-9.7	3.8	1.3	ADH1B	alcohol dehydrogenase IB (class I), beta polypeptide	NM_000668
P01134_D11	-1.7	2	1.3	-3.6	1.6	1.2	ADH1C	alcohol dehydrogenase 1C (class I), gamma polypeptide	NM_000669
P01070_D05	-1.3	-1.4	1.2	-2.2	1.7	1.1	ADH5	alcohol dehydrogenase 5 (class III), chi polypeptide	NM_000671
P01094_D10	-2.3	2.5	1.1	-2.2	1.8	-1	ADORA2B	adenosine A2b receptor	NM_000676
P01124_F09	-1.5	1.6	1.1	-1.8	1.9	1	AHR	aryl hydrocarbon receptor	NM_001621
P01101_B03	-2.4	1	1	-3	2.8	1.1	AKAP2	A kinase (PRKA) anchor protein 2	NM_007203
P01120_C03	-1.9	2	1.2	-1.2	1.5	1.2	AKR1B1	aldo-keto reductase family 1, member B1 (aldose reductase)	NM_001628
P01134_B08	-2.7	2.6	1.1	-1.4	1.9	1.2	AKR1B10	aldo-keto reductase family 1, member B10 (aldose reductase)	NM_020299

P01069_C01	-2.8	3.1	1.2	-2.2	2.6	1.1	AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	NM_001353
P01081_A11	-2.3	3.3	1.6	-2.2	1.9	1.3	AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	NM_001354
P01143_D10	-2.8	3.2	1.3	-2.1	2.7	1.1	AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	NM_001354
P01106_C11	-2.3	2.8	1.2	-2	2.5	1.1	AKR1C3	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	NM_003739
P01094_D12	-2.8	3.6	1.2	-2.5	1.7	1.2	ALDH1A3	aldehyde dehydrogenase 1 family, member A3	NM_000693
P01094_E01	-1.4	1.8	1.1	-2.1	1.6	1.1	ALDH3A2	aldehyde dehydrogenase 3 family, member A2	NM_000382
P01140_G11	-1.9	2.7	1.4	-2.5	1.8	1.1	ALDH3A2	aldehyde dehydrogenase 3 family, member A2	NM_000382
P01118_A12	-1.9	1.6	1.1	-2.6	2.2	1	ALEX1	ALEX1 protein	NM_016608
P01096_E12	-2.4	2	1	-2.1	2.2	1	ANG	angiogenin, ribonuclease, RNase A family, 5	NM_001145
P01145_E08	-2	2.3	1.2	-2.9	2.6	-1	ANGPT1	angiopoietin 1	NM_001146
P01091_G02	-1.2	1.5	1.2	-2.7	2	1.1	ANGPT2	angiopoietin 2	NM_001147
P01094_D06	-2.1	1.9	-1	-1.9	1.3	-1.1	ANK3	ankyrin 3, node of Ranvier (ankyrin G)	NM_001149
P01128_A07	-1.5	1.8	1.2	-2.2	2.3	1.2	AOX1	aldehyde oxidase 1	NM_001159
P01116_H05	-1.1	1.4	1.2	-2	1.8	1	APELIN	apelin; peptide ligand for APJ receptor	NM_017413
P01103_F06	2.4	-2.4	-1.1	1.4	-1.5	-1.1	APG3	autophagy Apg3p/Aut1p-like	NM_022488
P01123_A07	3.2	-3	-1	1.5	-1.8	-1	APOA1	apolipoprotein A-I	NM_000039
P01105_G06	-2.2	1.8	-1.1	-4.5	5.7	1.1	APOC1	apolipoprotein C-I	NM_001645
P01124_G03	-1.3	1.4	1	-2.4	2	1.1	APOE	apolipoprotein E	NM_000041
P01105_B02	-1.6	1.8	-1	-2.9	1.9	1.2	ARHGAP6	Rho GTPase activating protein 6	NM_001174
P01064_G03	-1.1	1.3	1.1	-2	1.6	1.2	ARHGEF16	Rho guanine exchange factor (GEF) 16	NM_014448
P01110_E10	-2	2.1	1.2	-2.3	1.9	1	ARHGEF3	Rho guanine nucleotide exchange factor (GEF) 3	NM_019555
P01142_C03	-1.6	1.8	1.5	-1.9	1.7	1.2	ARHI	ras homolog gene family, member I	NM_004675
P01138_A09	1.9	-2.2	-1.1	1.8	-1.9	-1.1	ARL4	ADP-ribosylation factor-like 4	NM_005738
P01064_G12	-1.7	1.8	1.1	-1.8	1.6	-1	ARNT2	aryl-hydrocarbon receptor nuclear translocator 2	NM_014862
P01088_H09	-1.5	1.7	1.2	-1.8	1.6	1.1	ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	NM_004315
P01105_F06	2.9	-2.8	1.1	2.1	-2.4	-1.2	ASNS	asparagine synthetase	NM_001673
P01070_E06	1.8	-1.5	-1.3	1.6	-1.4	1	ATF3	activating transcription factor 3	NM_001674
P01122_G07	-1.2	1.7	1.2	-1.8	1.5	1.3	AXIN2	axin 2 (conductin, axil)	NM_004655
P01115_D06	-1.4	1.6	1	-2	1.5	-1.1	B3GALT2	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2	NM_003783
P01128_A08	-1.6	1.7	1	-2.4	1.7	-1	B3GALT3	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase,	NM_003781

								polypeptide 3	
P01095_F06	2.4	-2.2	1.1	1.3	-1.5	-1	BAI3	brain-specific angiogenesis inhibitor 3	NM_001704
P01094_C02	-1.8	2	1.2	-2.4	2.7	-1	BF	B-factor, properdin	NM_001710
P01134_E02	-1.7	1.8	1	-2.2	1.6	-1	BFSP1	beaded filament structural protein 1, filensin	NM_001195
P01081_D08	-1.2	1.7	1.2	-3.5	1.8	1.2	BIRC1	baculoviral IAP repeat-containing 1	NM_004536
P01094_B06	-2.6	2.9	1.1	-4	2.5	-1	BMP4	bone morphogenetic protein 4	NM_001202
P01145_A02	-3.2	2.3	1	-3.6	3.2	-1.1	BNIP2	BCL2/adenovirus E1B 19kDa interacting protein 2	NM_004330
P01075_F05	-1.5	1.5	1.2	-1.8	2	1.2	BRE	brain and reproductive organ-expressed (TNFRSF1A modulator)	NM_004899
P01124_B10	-1.3	1.5	1.3	-2.2	1.6	1.2	BST1	bone marrow stromal cell antigen 1	NM_004334
P01094_B08	-1.8	1.6	-1.1	-1.2	1.3	-1.1	BTD	biotinidase	NM_000060
P01093_E08	-2	1.5	-1.1	-1.9	2.7	1.1	C1R	complement component 1, r subcomponent	NM_001733
P01077_E12	-1.4	1.6	1.1	-1.8	1.9	-1.1	C1S	complement component 1, s subcomponent	NM_001734
P01097_G03	1.9	-1.7	-1	1	-1.5	-1.1	C20orf14	chromosome 20 open reading frame 14	NM_012469
P01140_A07	2.3	-3.2	-1	3	-2.6	-1	C20orf97	chromosome 20 open reading frame 97	NM_021158
P01069_E02	-1.7	1.6	1.1	-3.3	3.2	1.1	C6	complement component 6	NM_000065
P01077_E10	-3.1	2.9	1.1	-8.2	4.7	-1	C7	complement component 7	NM_000587
P01099_C10	-1.8	2.1	1.2	-2.7	3.5	1.1	CA12	carbonic anhydrase XII	NM_001218
P01117_G05	-3	2.4	-1.1	-2.2	2.3	1.1	CAMK2B	calcium/calmodulin-dependent protein kinase (CaM kinase) II beta	NM_001220
P01114_A05	-2.7	3.9	1.2	-3.5	2.7	1	CAMK2D	calcium/calmodulin-dependent protein kinase (CaM kinase) II delta	NM_001221
P01080_B05	-2.3	3	1.1	-2.3	2.1	1.1	CAMK2D	calcium/calmodulin-dependent protein kinase (CaM kinase) II delta	NM_001221
P01063_E07	-1.6	2	1.2	-1.8	1.6	1.1	CASP1	caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)	NM_001223
P01093_G08	-2.4	2.3	-1.2	-2.1	2.4	1	CAV1	caveolin 1, caveolae protein, 22kDa	NM_001753
P01093_E04	1.8	-1.7	-1.1	1.6	-1.9	-1.1	CBS	cystathionine-beta-synthase	NM_000071
P01064_D02	-1.5	1.6	-1.3	-2.2	2.8	-1.1	CCL13	chemokine (C-C motif) ligand 13	NM_005408
P01072_E08	-1.3	1.4	-1.2	-2.2	3.2	-1.1	CCL7	chemokine (C-C motif) ligand 7	NM_006273
P01127_H03	1.1	1.2	-1.3	-2	2.9	-1	CCL8	chemokine (C-C motif) ligand 8	NM_005623
P01070_A04	-1.4	1.9	1.2	-3.2	2.4	1.1	CCR2	chemokine (C-C motif) receptor 2	NM_000647
P01138_B02	-1.2	1.3	1.3	-3.6	1.5	1	CCRL1	chemokine (C-C motif) receptor-like 1	NM_016557
P01069_H09	-1.9	1.9	1.3	-3.6	1.8	1.2	CD36	CD36 antigen (collagen type I receptor, thrombospondin receptor)	NM_000072
P01072_E03	-2.8	2.7	1.2	-2.9	2.8	1.2	CDC25B	cell division cycle 25B	NM_004358
P01093_H07	2	-4.3	1.2	2.1	-2	-1	CDH2	cadherin 2, type 1, N-cadherin (neuronal)	NM_001792
P01129_E07	1.7	-1.4	1.1	2	-1.9	-1.1	CDH4	cadherin 4, type 1, R-cadherin (retinal)	NM_001794

P01130_H07	2.1	-2.4	-1.1	1.9	-1.8	-1	CDH5	cadherin 5, type 2, VE-cadherin (vascular epithelium)	NM_001795
P01116_H02	-3.3	2.1	1.1	-2	2.4	1.1	CDK5RAP2	CDK5 regulatory subunit associated protein 2	NM_018249
P01102_B02	-2.1	2.5	1	-3.4	3.2	-1.1	CDSN	corneodesmosin	NM_001264
P01140_G02	-1.4	1.3	1.1	-2.9	2.4	1	CEACAM5	carcinoembryonic antigen-related cell adhesion molecule 5	NM_004363
P01094_A06	-1.6	1.3	1.3	-4.2	2.9	1	CEACAM5	carcinoembryonic antigen-related cell adhesion molecule 5	NM_004363
P01062_G02	-1.3	1.5	1.3	-2.9	2.1	1.1	CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)	NM_002483
P01099_B05	-1.8	1.8	1.1	-2.9	3	-1.1	CEACAM7	carcinoembryonic antigen-related cell adhesion molecule 7	NM_006890
P01090_E04	-1.3	1.6	1.3	-1.9	1.8	-1	CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	NM_005195
P01070_A01	-2.6	3.1	-1	-9.2	9.2	1.1	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	NM_001276
P01125_G02	-2.9	2	1	-5	6.2	1	CHI3L2	chitinase 3-like 2	NM_004000
P01134_F10	8	-6.3	1.2	19.5	-8	1.1	CILP	cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	NM_003613
P01089_A12	-1.9	2.1	1	-2.1	2.1	-1	CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	NM_006079
P01076_A07	2.1	-1.8	-1	1.4	-1.2	1.1	CKAP4	cytoskeleton-associated protein 4	NM_006825
P01104_C09	2.2	-2.4	1.1	4.3	-2.8	1	CKLF	chemokine-like factor	NM_016326
P01103_G05	-1.4	1.6	1.3	-2.5	1.5	1.3	CLDN1	claudin 1	NM_021101
P01105_D03	-3	2.7	1.3	-2.6	2	-1	CLECSF2	C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 2 (activation-induced)	NM_005127
P01064_F09	2.2	-1.5	1.2	1.2	-1.2	1.1	CNN1	calponin 1, basic, smooth muscle	NM_001299
P01090_A03	-1.1	1.3	1.2	-2.2	1.6	1.1	CNTNAP1	contactin associated protein 1	NM_003632
P01069_F02	1.2	1.2	1.2	3.2	-3	1	COL15A1	collagen, type XV, alpha 1	NM_001855
P01077_E08	1.8	-1.5	1	1.9	-1.9	-1	COL1A2	collagen, type I, alpha 2	NM_000089
P01093_F03	1.7	-2.3	1	1.9	-2.1	-1	COL4A2	collagen, type IV, alpha 2	NM_001846
P01105_C12	1.8	-1.5	1.4	3.1	-2.3	1.1	COL7A1	collagen, type VII, alpha 1 (epidermolysis bullosa, dystrophic, dominant and recessive)	NM_000094
P01120_G04	2.7	-2.1	1.2	3.8	-3.6	1.1	COL8A2	collagen, type VIII, alpha 2	M60832
P01084_A12	-4.9	4.7	1.1	-9.9	6	1	COLEC12	collectin sub-family member 12	NM_030781
P01082_H06	1.3	-1.3	1.2	3.3	-2.4	1.2	COMP	cartilage oligomeric matrix protein (pseudoachondroplasia, epiphyseal dysplasia 1, multiple)	NM_000095
P01129_C12	1.4	-1.5	1.3	2.6	-1.6	1.3	COMP	cartilage oligomeric matrix protein (pseudoachondroplasia, epiphyseal dysplasia 1, multiple)	NM_000095
P01076_C09	-2.2	2.7	1.2	-2.1	1.6	1.1	COPB	coatomer protein complex, subunit beta	NM_016451

P01085_D11	-4	4.2	1.1	-7.7	4.1	1.1	CPA4	carboxypeptidase A4	NM_016352
P01104_A07	-1.9	2	1.2	-2.5	2.2	1	CPD	carboxypeptidase D	NM_001304
P01077_G01	1.9	-1.8	1.1	1.7	-1.9	1.1	CRABP2	cellular retinoic acid binding protein 2	NM_001878
P01095_E03	-1.8	1.8	1.2	-2.1	2	-1.1	CREG	cellular repressor of E1A-stimulated genes	NM_003851
P01124_E01	-2.2	2.1	1	-2.5	2.2	-1	CREM	cAMP responsive element modulator	NM_001881
P01120_B01	1.8	-1.6	1.2	3.9	-3.4	1.1	CRLF1	cytokine receptor-like factor 1	NM_004750
P01120_D10	-1.5	1.9	1.3	-3.5	2.4	1.1	CROT	carnitine O-octanoyltransferase	NM_021151
P01124_F10	-1.2	1.3	1.2	-1.8	1.7	1.1	CRYAA	crystallin, alpha A	NM_000394
P00777_A08	-2	1.6	1.1	-2.6	2.5	-1.1	CRYAB	crystallin, alpha B	NM_001885
P01077_E04	-2.1	1.8	1.1	-2.5	2.6	-1.1	CRYAB	crystallin, alpha B	NM_001885
P01125_B11	-1.8	1.2	1.1	-1.8	1.8	1	CSF1	colony stimulating factor 1 (macrophage)	NM_000757
P01108_G05	3.8	-3	1.1	2.3	-2.4	1	CSPG2	chondroitin sulfate proteoglycan 2 (versican)	NM_004385
P01075_F12	-1.5	1.6	1.1	-2	2	1.1	CSRP2	cysteine and glycine-rich protein 2	NM_001321
P01145_A03	-2.1	2.4	1	-3.7	3.4	-1.1	CST4	cystatin S	NM_001899
P00777_D03	2.5	-2	1.1	1.1	-1.4	-1.2	CTGF	connective tissue growth factor	NM_001901
P01077_D08	2.6	-3.5	-1.2	1.8	-2.7	-1.2	CTGF	connective tissue growth factor	NM_001901
P01069_D11	2	-2.1	1.2	1.9	-1.4	1.2	CTH	cystathionase (cystathionine gamma-lyase)	NM_001902
P01099_B01	-1.7	2	1.1	-2	1.6	-1	CTNNAL1	catenin (cadherin-associated protein), alpha-like 1	NM_003798
P01093_G10	-1.4	1.4	1.1	-1.8	2	1	CTSC	cathepsin C	NM_001814
P01077_G03	-1	1.3	1.2	-1.8	1.7	1.2	CTSH	cathepsin H	NM_004390
P01069_H12	-1.5	1.5	1.1	-2.3	2.6	-1	CTSK	cathepsin K (pycnodysostosis)	NM_000396
P01093_G09	-2.5	2.1	1.1	-2	2.3	-1	CTSL	cathepsin L	NM_001912
P01112_D02	-1.6	1.8	1.2	-2.9	2.1	-1	CUGBP2	CUG triplet repeat, RNA binding protein 2	NM_006561
P01131_G04	-1.3	1.6	1.3	-2.2	1.7	1.3	CUGBP2	CUG triplet repeat, RNA binding protein 2	NM_006561
P01090_H01	-2	1.8	1.3	-1.5	1.9	1.3	CUL5	cullin 5	NM_003478
P01085_C05	-3.8	3.4	1.1	-5.5	5	-1	CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	NM_001511
P01093_A02	-3.7	3.1	1	-5.8	5.4	-1	CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	NM_001511
P01125_H11	-2.4	2	1.1	-2.3	2.1	1	CXCL3	chemokine (C-X-C motif) ligand 3	NM_002090
P01136_B01	-4.5	4.4	1.1	-8.4	10	1.1	CXCL6	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	NM_002993
P01069_D07	-2.4	2.3	1.3	-2	1.7	1	CYB5	cytochrome b-5	NM_001914
P00777_A11	2	-2.5	-1	1.8	-2	-1	CYR61	cysteine-rich, angiogenic inducer, 61	NM_001554
P00777_C11	1.8	-2.5	-1	1.8	-1.9	-1.1	CYR61	cysteine-rich, angiogenic inducer, 61	NM_001554
P00777_C12	2	-2.6	-1.1	1.9	-1.9	-1.1	CYR61	cysteine-rich, angiogenic inducer, 61	NM_001554
P01108_B04	2.3	-2.4	1.1	1.9	-1.9	-1.1	CYR61	cysteine-rich, angiogenic inducer, 61	NM_001554
P01130_H03	2	-2.4	-1.1	1.9	-1.8	-1.1	CYR61	cysteine-rich, angiogenic inducer, 61	NM_001554
P01100_C06	2.2	-2.4	-1.1	1.8	-1.9	-1	DACT1	dapper homolog 1, antagonist of beta-catenin (xenopus)	NM_016651

P01069_C07	1.7	-1.4	1.1	2.3	-2.1	1.1	DAF	decay accelerating factor for complement (CD55, Cromer blood group system)	NM_000574
P01129_B04	-2.8	2.5	1.2	-4.3	3.5	1	DAPK1	death-associated protein kinase 1	NM_004938
P01092_G02	-2.7	2.6	1.1	-3.8	2.8	1.2	DAPK1	death-associated protein kinase 1	NM_004938
P01065_A02	-1.8	1.9	-1	-1.8	1.7	-1.1	DDX38	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 38	NM_014003
P01105_A10	-3.7	2.9	-1	-7	5.4	-1	DKK1	dickkopf homolog 1 (Xenopus laevis)	NM_012242
P01113_E05	2.8	-2.4	-1.1	1.8	-2.1	-1.1	DLC1	deleted in liver cancer 1	NM_006094
P01093_C11	-1.8	1.9	-1	-4.5	3.3	-1	DPP4	dipeptidylpeptidase 4 (CD26, adenosine deaminase complexing protein 2)	NM_001935
P01073_G11	-1.8	1.7	1	-1.6	1.5	1	DPYSL2	dihydropyrimidinase-like 2	NM_001386
P01090_F08	1.4	-1.5	-1.1	2	-1.9	-1.1	DSCR1	Down syndrome critical region gene 1	NM_004414
P01122_D11	1.7	-1.2	1.3	1.9	-2.1	1.3	EBAF	endometrial bleeding associated factor (left-right determination, factor A; transforming growth factor beta superfamily)	NM_003240
P01123_B11	-1.8	1.7	1	-2.3	1.9	1	ECM2	extracellular matrix protein 2, female organ and adipocyte specific	NM_001393
P01124_E11	-1.6	1.9	1.2	-2.1	1.9	1.2	EDG1	endothelial differentiation, sphingolipid G-protein-coupled receptor, 1	NM_001400
P01103_G08	-1.8	1.8	1.1	-2.4	2.4	1	EDG2	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	NM_001401
P01093_C01	-2.1	1.5	-1.1	-2.9	1.9	-1.3	EDN1	endothelin 1	NM_001955
P01105_H10	-1.9	1.9	1	-2.2	2.3	-1	EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	NM_004105
P01064_A03	-1.4	1.9	1.2	-2	2	1.1	EFNB3	ephrin-B3	NM_001406
P01093_B07	-1.8	1.7	1.3	-1.5	1.3	1.1	EGR2	early growth response 2 (Krox-20 homolog, Drosophila)	NM_000399
P01121_C03	-2	2	1.2	-1.2	1.5	1.2	EHD3	EH-domain containing 3	NM_014600
P01065_E02	1.9	-1.6	1.2	3.4	-3.3	1.1	ELN	elastin (supravalvular aortic stenosis, Williams-Beuren syndrome)	NM_000501
P01096_H11	-3.4	3.7	1.1	-3.5	3.2	-1	EPAS1	endothelial PAS domain protein 1	NM_001430
P01102_E11	-2	2.1	1.2	-2.5	2.1	-1	EPB41L2	erythrocyte membrane protein band 4.1-like 2	NM_001431
P01104_A05	-2.3	3.3	1.1	-2.2	2.3	1.1	EPI64	EBP50-PDZ interactor of 64 kD	NM_031937
P01130_H01	-2	2	1.1	-2.5	3.9	-1	EPOR	erythropoietin receptor	NM_000121
P01077_A07	-1.6	2.7	1.4	-2.3	2.1	1.2	ETV5	ets variant gene 5 (ets-related molecule)	NM_004454
P01097_C06	-5.9	4.9	-1	-15.8	14	-1.1	EVI2B	ecotropic viral integration site 2B	NM_006495
P01077_A01	1.8	-1.8	1.1	1.3	-1.4	1	EXT1	exostoses (multiple) 1	NM_000127
P01069_F04	-1.7	1.6	1.2	-2.1	1.7	1.1	F2R	coagulation factor II (thrombin) receptor	NM_001992
P01128_B02	1.8	-1.9	1.1	-1	-1.1	-1	F3	coagulation factor III (thromboplastin, tissue factor)	NM_001993
P01132_G03	1.9	-1.7	1.2	1.8	-1.6	1.1	FACL3	fatty-acid-Coenzyme A ligase, long-chain 3	NM_004457
P01096_A03	1.8	-2	1	1.8	-2	1	FACL3	fatty-acid-Coenzyme A ligase, long-chain 3	NM_004457

P01083_D07	2.2	-1.6	1.2	1.4	-1.3	1	FADS1	fatty acid desaturase 1	NM_013402
P01093_B02	-2	1.6	1.1	-3.4	3.4	1	FBLN1	fibulin 1	NM_001996
P01123_A08	3.4	-3	1.2	1.6	-1.9	-1	FBLN5	fibulin 5	NM_006329
P01068_H09	1.4	-1.4	1	2.2	-2	-1	FBN1	fibrillin 1 (Marfan syndrome)	NM_000138
P01084_E10	1.9	-1.7	1.3	-1.1	-1.1	1.1	FGF18	fibroblast growth factor 18	NM_003862
P01093_B03	-4.2	4.9	1.2	-5.9	5.6	1	FGF7	fibroblast growth factor 7 (keratinocyte growth factor)	NM_002009
P01092_C04	-3.2	2.9	1.1	-3.1	2.3	-1	FGL2	fibrinogen-like 2	NM_006682
P01126_F06	-5	5.2	-1.1	-6.5	4.9	-1.1	FMO2	flavin containing monooxygenase 2	NM_001460
P01078_G11	-1.9	2.1	1.2	-3.1	2.2	1.1	FMO3	flavin containing monooxygenase 3	NM_006894
P01088_F09	2	-1.8	1.2	1.4	-1.5	1.1	FOXD1	forkhead box D1	NM_004472
P01120_B03	-1.8	1.9	1.3	-1.2	1.5	1.2	FRA	Fos-related antigen	NM_024816
P01138_B06	-1.8	1.5	1	-1.4	1.8	-1	FTHL17	ferritin, heavy polypeptide-like 17	NM_031894
P01068_G11	2.7	-2.1	1.3	2.4	-2.5	1.1	FUT4	fucosyltransferase 4 (alpha (1,3) fucosyltransferase, myeloid-specific)	NM_002033
P01077_A05	1.8	-1.5	1	1.5	-1.2	1	FYN	FYN oncogene related to SRC, FGR, YES	NM_002037
P01124_G01	-1.9	1.9	1.1	-1.4	1.2	1	FZD7	frizzled homolog 7 (Drosophila)	NM_003507
P01083_B09	3.2	-4	-1	4.2	-3.5	-1	GABARAPL2	GABA(A) receptor-associated protein-like 2	NM_007285
P01106_B05	-1.8	1.5	1	-1.1	2.4	1.2	GALT	galactose-1-phosphate uridylyltransferase	NM_000155
P01092_G07	2.5	-2.3	-1.2	1.8	-2.2	-1.1	GARS	glycyl-tRNA synthetase	NM_002047
P01085_D09	-2.9	4.1	1.4	-5.3	2.6	1.3	GAS1	growth arrest-specific 1	NM_002048
P01063_E09	-2	1.7	1.1	-2	1.8	1.2	GBP2	guanylate binding protein 2, interferon-inducible	NM_004120
P01123_D12	-1.9	1.4	-1.2	-2.7	2.7	-1.1	GBP2	guanylate binding protein 2, interferon-inducible	NM_004120
P01135_C03	-1.8	1.9	1.2	-2.7	2.4	1.1	GCNT1	glucosaminyl (N-acetyl) transferase 1, core 2 (beta-1,6-N-acetylglucosaminyltransferase)	NM_001490
P01127_B01	-2.8	2.2	-1.1	-2.9	3.7	-1	GDF5	growth differentiation factor 5 (cartilage-derived morphogenetic protein-1)	NM_000557
P01065_A06	-1.7	1.7	1.1	-1.8	1.6	1	GGA3	golgi associated, gamma adaptin ear containing, ARF binding protein 3	NM_014001
P01076_H05	-2	2.4	1.2	-2.7	1.9	1.1	GM2A	GM2 ganglioside activator protein	NM_000405
P01062_E04	-2.3	1.9	-1	-2.1	1.9	-1	GNPI	glucosamine-6-phosphate isomerase	NM_005471
P01138_C10	-2.1	2.2	-1.1	-2	1.9	-1.1	GNPI	glucosamine-6-phosphate isomerase	NM_005471
P01074_D06	3.5	-4	-1	5.7	-3.6	1.1	GOLGA4	golgi autoantigen, golgin subfamily a, 4	NM_002078
P01083_C04	-1.1	1.2	1.2	-1.8	1.5	1.1	GOLPH2	golgi phosphoprotein 2	NM_016548
P01125_G10	1.8	-1.9	-1	1.6	-1.9	-1.1	GOLPH4	golgi phosphoprotein 4	NM_014498
P01131_F08	1.7	-2.3	-1.2	1.8	-1.6	-1.2	GOT1	glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	NM_002079
P01080_A01	-1.2	1.9	1.3	-3.6	1.8	1.2	GPM6B	glycoprotein M6B	NM_005278
P01082_E09	-2.2	2.3	1.2	-2.9	2.3	1	GPNMB	glycoprotein (transmembrane) nmb	NM_002510
P01087_G08	-3	2.3	1	-4.9	4	-1	GPNMB	glycoprotein (transmembrane) nmb	NM_002510
P01140_E04	-1.8	1.6	1.3	-2.5	1.8	1.1	GPRK5	G protein-coupled receptor	NM_005308

								kinase 5	
P01068_E08	-3.2	1.8	-1.1	-1.9	2.9	1.1	GSTM1	glutathione S-transferase M1	NM_000561
P01068_E09	-1.8	1.5	1.1	-1.7	2.3	1.1	GSTM3	glutathione S-transferase M3 (brain)	NM_000849
P01086_A10	-2.4	1.5	1.1	-1.9	2.7	1.1	GSTM5	glutathione S-transferase M5	NM_000851
P01080_C03	1.7	-1.8	1.1	2.1	-1.9	-1	GTPBP2	GTP binding protein 2	NM_019096
P01108_A05	-1.2	1.5	1.2	-1.9	1.7	1.1	GYPC	glycophorin C (Gerbich blood group)	NM_002101
P01121_B02	-1.6	1.2	-1	-1.9	1.9	1.1	HAGE	DEAD-box protein	NM_018665
P01133_H11	-1.2	1.8	1.4	-2.1	1.7	1.2	HAS2	hyaluronan synthase 2	NM_005328
P01101_C10	-1.8	1.6	-1	-1.4	1.5	-1	HEBP1	heme binding protein 1	NM_015987
P01137_B02	1.8	-1.5	1	1.6	-1.3	1	HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	NM_014685
P01136_A05	2	-2.1	1.1	2	-1.8	-1	HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	NM_014685
P01083_G12	1.6	-1.1	1.1	2.2	-1.5	1.2	HEYL	hairy/enhancer-of-split related with YRPW motif-like	NM_014571
P01126_B01	-1.3	1.4	1.1	-1.8	1.8	-1	HFL1	H factor (complement)-like 1	NM_002113
P01075_H10	-3.6	6.2	1.3	-5.3	3.8	1.3	HGF	hepatocyte growth factor (hepapoietin A; scatter factor)	NM_000601
P01110_C10	1.9	-1.6	1.3	1.3	-1.2	1.1	HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	NM_000859
P01112_G07	2	-1.7	1.3	-1	-1.1	-1	HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	NM_002130
P01064_F02	-2	2.6	1.1	-3.1	3	1	HNMT	histamine N-methyltransferase	NM_006895
P01078_F05	-2.1	2.4	1.2	-2.1	2.6	1.1	HPN	hepsin (transmembrane protease, serine 1)	NM_002151
P01107_H06	1.8	-1.9	-1.2	1.4	-1.7	-1.3	IARS	isoleucine-tRNA synthetase	NM_002161
P01100_C10	-1.2	1.5	1.3	-1.8	1.7	1.1	ICOS	inducible T-cell co-stimulator	NM_012092
P01124_A06	-1.7	2	1.3	-1.8	1.7	-1	ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	NM_002166
P01072_H03	1.8	-1.6	1.1	1.6	-1.6	1.2	ID4	inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	NM_001546
P01088_C01	-2.4	2.2	1	-2.5	2.2	-1	IDH2	isocitrate dehydrogenase 2 (NADP+), mitochondrial	NM_002168
P01130_F01	4.5	-2.9	1.3	1.8	-1.7	1	IGF1	insulin-like growth factor 1 (somatomedin C)	NM_000618
P01063_D10	2.1	1	1.2	3.8	-1.9	1.3	IGF1	insulin-like growth factor 1 (somatomedin C)	NM_000618
P00777_D09	-2.6	2.2	1.1	-2.9	3.2	1.2	IGFBP4	insulin-like growth factor binding protein 4	NM_001552
P01130_B02	12.3	-11.1	1.2	6.1	-5.4	1.1	IL11	interleukin 11	NM_000641
P01088_D05	-2	2	1.2	-1.8	1.4	1.1	IL1B	interleukin 1, beta	NM_000576
P01063_E06	-3	3.3	1.1	-6.7	6.1	1.1	IL1R1	interleukin 1 receptor, type I	NM_000877
P01110_E12	-1.4	2.3	1.3	-2.5	1.5	1.1	IL1R1	interleukin 1 receptor, type I	NM_000877
P01145_A04	-3	2.4	-1	-4.2	2.7	-1	IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	NM_002184
P01091_B03	-1.9	1.9	-1	-1.3	1.3	1	IMPA2	inositol(myo)-1(or 4)-monophosphatase 2	NM_014214
P01063_E03	1.7	-1.7	-1.2	2.4	-1.7	1.1	INDO	indoleamine-pyrrole 2,3 dioxygenase	NM_002164
P01082_F07	2.1	-2.6	-1.1	2.2	-1.5	1.2	INHBA	inhibin, beta A (activin A, activin AB alpha polypeptide)	NM_002192

P01130_D09	2.1	-1.7	-1	1.7	-1.7	-1.1	INPP4B	inositol polyphosphate-4-phosphatase, type II, 105kDa	NM_003866
P01067_B04	2	-1.7	1.2	1.6	-1.3	1.1	INSIG1	insulin induced gene 1	NM_005542
P01074_G10	-1.7	1.7	-1	-4.7	3.1	-1.1	IQGAP2	IQ motif containing GTPase activating protein 2	NM_006633
P01061_E02	2.6	-2.6	1	2.4	-2.5	-1	ISGF3G	interferon-stimulated transcription factor 3, gamma 48kDa	NM_006084
P01140_B08	1.8	-1.7	1.2	3	-1.8	1	ITGA11	integrin, alpha 11	NM_012211
P01088_C11	-1.5	1.8	1.2	-1.8	1.9	1.1	ITGAM	integrin, alpha M (complement component receptor 3, alpha; also known as CD11b (p170), macrophage antigen alpha polypeptide)	NM_000632
P01081_E02	2.3	-1.8	1.2	2.2	-2.2	1	JUNB	jun B proto-oncogene	NM_002229
P01072_G01	1.6	-1.5	1.2	1.9	-1.6	1.1	JUP	junction plakoglobin	NM_002230
P01079_A01	-1.9	2.1	-1.1	-1.5	1.5	-1	JWA	vitamin A responsive; cytoskeleton related	NM_006407
P01122_A09	1.1	1.3	1.2	-1.9	1.6	1.1	KCNE3	potassium voltage-gated channel, Isk-related family, member 3	NM_005472
P01113_F02	-1.8	1.9	1.2	-2.4	2.3	1.1	KHDRBS3	KH domain containing, RNA binding, signal transduction associated 3	NM_006558
P01074_B01	-1.6	1.2	1.1	-1.9	1.7	1	KIAA0102	KIAA0102 gene product	NM_014752
P01104_A04	-3.2	3.8	-1	-3	3.4	-1	KIAA1049	KIAA1049 protein	NM_014972
P01120_B02	-1.6	1.5	1.1	-1.8	1.7	1	KIF1B	kinesin family member 1B	NM_015074
P01088_C06	-1.6	1.6	1.2	-1.9	1.8	1	KRT4	keratin 4	NM_002272
P01085_D06	-1.8	1.7	1.2	-3.8	4.1	1	LAMA4	laminin, alpha 4	NM_002290
P01131_H02	-1.4	1.4	1.1	-2	1.9	-1.1	LAMC1	laminin, gamma 1 (formerly LAMB2)	NM_002293
P01131_H10	-2.4	1.8	-1.1	-2.1	1.5	1	LCN2	lipocalin 2 (oncogene 24p3)	NM_005564
P01100_H05	-2.8	2.7	1.2	-5	2.7	1	LEPR	leptin receptor	NM_002303
P01088_B02	-2.3	2.4	1.1	-2.6	2.1	-1	LGALS3	lectin, galactoside-binding, soluble, 3 (galectin 3)	NM_002306
P01081_B11	-3.5	1.3	1.1	-4.6	4.4	1	LHFP	lipoma HMGIC fusion partner	NM_005780
P01107_D06	2.2	-2	-1	1.7	-1.8	-1.1	LIMK2	LIM domain kinase 2	NM_005569
P01085_G06	1.2	-1.4	-1.1	1.9	-2.1	-1	LMO7	LIM domain only 7	NM_005358
P01085_D05	-2.1	2.2	1.2	-3.9	3.7	1.1	LOC56270	hypothetical protein 628	NM_019613
P01082_E01	2.1	-1.5	1.2	1.8	-1.6	1.2	LOX	lysyl oxidase	NM_002317
P01083_H02	-1.4	1.5	1.1	-2	2	1	LPHN2	latrophilin 2	NM_012302
P01131_D06	-1.6	1.7	1.2	-2.4	1.8	1.2	LRP4	low density lipoprotein receptor-related protein 4	AB011540
P01072_F03	1.8	-1.2	-1	2.2	-1.6	-1	LTBP2	latent transforming growth factor beta binding protein 2	NM_000428
P01088_C04	-2.3	2.3	1.1	-4.4	4.7	1.1	LTF	lactotransferrin	NM_002343
P01063_A11	-2.3	2.4	-1	-4.8	3.9	-1	LUM	lumican	NM_002345
P01135_G05	-2.4	2.4	1.2	-1.7	1.6	-1	LY96	lymphocyte antigen 96	NM_015364
P01085_C04	-2	1.8	1.2	-2	1.5	1	MADH3	MAD, mothers against decapentaplegic homolog 3 (Drosophila)	NM_005902
P01091_G10	1.8	-1.4	1.2	2.2	-2.1	1.2	MADH7	MAD, mothers against decapentaplegic homolog 7 (Drosophila)	NM_005904
P01089_C01	1.2	-1.2	-1	1.8	-1.6	-1.2	MAGP2	Microfibril-associated glycoprotein-2	NM_003480
P01084_A09	1.8	-1.6	1.2	1.4	-1.6	-1	MAP3K2	mitogen-activated protein kinase kinase kinase 2	NM_006609
P01073_E08	-2	2.4	-1	-2.3	1.8	-1	MAP3K5	mitogen-activated protein kinase kinase kinase 5	NM_005923

P01066_F10	2	-2	1.1	1.9	-1.7	1.1	MAPK7	mitogen-activated protein kinase 7	NM_002749
P01076_B12	1.9	-2.1	-1.1	1.7	-1.7	-1.1	MAPRE2	microtubule-associated protein, RP/EB family, member 2	NM_014268
P01134_C04	3.1	-2.1	1.1	2.8	-3.3	-1	MATN3	matrilin 3	NM_002381
P01145_A05	-1.7	1.9	-1	-2.6	2.1	-1	ME1	malic enzyme 1, NADP(+)-dependent, cytosolic	NM_002395
P01072_D11	-3.3	3.7	-1	-3.5	3.1	1	MEST	mesoderm specific transcript homolog (mouse)	NM_002402
P01121_F04	-1.9	2.1	1.3	-2.1	1.8	1	MGC1203	hypothetical protein MGC1203	NM_024296
P01068_F12	-2.9	2.8	1.1	-2.6	2.4	-1	MGST1	microsomal glutathione S-transferase 1	NM_020300
P01091_B06	-1.8	1.6	-1	-1.5	1.6	-1.1	MGST2	microsomal glutathione S-transferase 2	NM_002413
P01099_H09	-2.4	2.3	1.1	-2.4	2	1.2	MID1	midline 1 (Opitz/BBB syndrome)	NM_000381
P01062_H05	-1.4	2.2	1.3	-2.4	2.4	1.3	MME	membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10)	NM_000902
P01125_H08	1	1	1.1	2.6	-2.1	-1	MMP11	matrix metalloproteinase 11 (stromelysin 3)	NM_005940
P01072_D02	2.8	-2.6	-1.3	1.7	-2	-1.2	MTHFD2	methylene tetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase	NM_006636
P01125_A10	-1.6	1.6	1.2	-1.8	1.5	1.1	MTMR4	myotubularin related protein 4	NM_004687
P01130_C09	1.9	-1.7	1.3	1.1	-1.1	1.1	MUCDHL	mucin and cadherin-like	NM_017717
P01102_A12	1.4	-1.3	1.2	2.5	-1.6	1.1	MVK	mevalonate kinase (mevalonic aciduria)	NM_000431
P01133_F05	1.9	-1.8	1	1.4	-1.4	-1.1	MYH9	myosin, heavy polypeptide 9, non-muscle	NM_002473
P01100_B07	-2	2.4	1.1	-5.5	2.6	-1.1	MYOZ2	myozenin 2	NM_016599
P01072_C06	-1.6	1.6	1	-2.6	2.6	1.1	NCK1	NCK adaptor protein 1	NM_006153
P01086_B12	-1.2	1.4	-1	-1.8	1.6	-1.1	NCOA3	nuclear receptor coactivator 3	NM_006534
P01135_C12	3.3	-3	1.3	3.1	-2.1	1.2	NEDD9	neural precursor cell expressed, developmentally down-regulated 9	NM_006403
P01112_A08	2.5	-2.1	1.2	1.7	-1.8	1.1	NET-6	transmembrane 4 superfamily member tetraspan NET-6	NM_014399
P01103_E02	-1.7	2.1	1.2	-2.5	2.1	-1	NFIA	nuclear factor I/A	AL096888
P01073_E06	-1.9	1.9	1	-2.1	1.8	-1.1	NFIB	nuclear factor I/B	NM_005596
P01064_C02	-1.9	2	1.2	-3.3	2.5	1	NID2	nidogen 2 (osteonidogen)	NM_007361
P01131_E08	2.3	-1.6	1.3	5.1	-3	1.3	NINJ2	ninjurin 2	NM_016533
P01072_D01	2.2	-2.2	1.1	2.2	-2.1	-1	NK4	natural killer cell transcript 4	NM_004221
P01121_G06	-2.2	2.1	-1.1	-2.5	2.2	-1.1	NOL3	nucleolar protein 3 (apoptosis repressor with CARD domain)	NM_003946
P01104_C08	6.9	-6.1	1.1	5.8	-5.8	1.1	NOX4	NADPH oxidase 4	NM_016931
P01107_D11	-1.7	1.6	-1	-1.8	1.8	1	NPC2	Niemann-Pick disease, type C2	NM_006432
P01132_G06	2.4	-2	1.3	1.5	-1.6	1.1	NPR3	natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)	NM_000908
P01096_F08	-1.5	1.6	1.2	-2.1	2	1.1	NPTX2	neuronal pentraxin II	NM_002523
P01126_E07	-1.5	2	1.2	-2	1.7	1.1	NR2F2	nuclear receptor subfamily 2, group F, member 2	NM_021005
P01064_G11	-1.5	1.6	1	-2.1	1.5	-1	NRCAM	neuronal cell adhesion molecule	NM_005010

P01097_E11	1.9	-1.8	1.1	1.6	-1.8	-1	NS1-BP	NS1-binding protein	NM_006469
P01103_C04	2.4	-2.2	-1	1.3	-1.5	-1	NUDT3	nudix (nucleoside diphosphate linked moiety X)-type motif 3	NM_006703
P01072_B11	2.6	-2.6	-1.1	2.3	-2.3	-1.2	ODC1	ornithine decarboxylase 1	NM_002539
P01082_E10	-1.4	2	1.3	-5.7	1.9	1.2	OGN	osteoglycin (osteoinductive factor, mimecan)	NM_014057
P01119_G07	-2.1	2.2	1.1	-2.2	1.6	-1	OSBPL1A	oxysterol binding protein-like 1A	NM_018030
P01075_F01	2.3	-1.6	-1	3.9	-3.7	-1	OSF-2	osteoblast specific factor 2 (fasciclin I-like)	NM_006475
P01129_A10	2.2	-1.6	-1	4.1	-3.6	-1	OSF-2	osteoblast specific factor 2 (fasciclin I-like)	NM_006475
P01126_B11	-2	1.5	-1.2	1.1	1.7	1.2	OXA1L	oxidase (cytochrome c) assembly 1-like	NM_005015
P01071_D09	-1.9	1.6	-1.1	1.1	1.7	1.3	OXA1L	oxidase (cytochrome c) assembly 1-like	NM_005015
P01085_C08	-1.3	1.3	1.1	-2.2	1.6	-1	OXTR	oxytocin receptor	NM_000916
P01125_D04	2.1	-1.7	1.2	4.2	-2.3	1.3	PACE4	paired basic amino acid cleaving system 4	NM_002570
P01090_D03	-1.4	1.2	-1	-2.4	1.8	-1.2	PARG1	PTPL1-associated RhoGAP 1	NM_004815
P01122_G06	2.6	-2.4	1.1	1.9	-2	-1	PAWR	PRKC, apoptosis, WT1, regulator	NM_002583
P01120_F04	-1.8	2.3	1.3	-2	1.7	1.1	PBF	papillomavirus regulatory factor PRF-1	NM_018660
P01071_G08	-2	1.5	-1	-1.4	1.7	1	PBP	prostatic binding protein	NM_002567
P01064_A09	1.1	-1.2	1.2	1.9	-1.5	1.2	PCDH1	protocadherin 1 (cadherin-like 1)	NM_002587
P01066_G05	-1.4	1.6	1.3	-3.2	2.4	1.2	PDE1A	phosphodiesterase 1A, calmodulin-dependent	NM_005019
P01128_B03	1.8	-1.7	1.1	-1.1	-1	-1	PDE5A	phosphodiesterase 5A, cGMP-specific	NM_001083
P01087_E02	3.4	-2.4	1.1	3	-3.7	-1	PDGFA	platelet-derived growth factor alpha polypeptide	NM_002607
P01081_F07	-2.3	2.1	1.1	-2.2	2.1	1.1	PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	NM_006206
P01142_D01	-1.1	-1.8	1.2	-2.2	1.9	1.2	PDGFRL	platelet-derived growth factor receptor-like	NM_006207
P01064_G02	1.3	-1.1	1.3	2.3	-2	1.2	PDGFRL	platelet-derived growth factor receptor-like	NM_006207
P01137_F04	-1.8	2	1.1	-2	1.4	1.1	PDP	pyruvate dehydrogenase phosphatase	NM_018444
P01071_H07	1.8	-1.9	1	1.3	-1	1.1	PFKP	phosphofructokinase, platelet	NM_002627
P01064_H07	-1.8	1.7	1	-1.6	1.5	1	PHF3	PHD finger protein 3	NM_015153
P01131_G12	1.2	-1	1.2	1.8	-1.7	1.2	PIGB	phosphatidylinositol glycan, class B	NM_004855
P01074_H07	-1.8	1.9	1.2	-1.9	1.6	1	PIK3R1	phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	AF279367
P01068_A02	-2.4	1.7	-1.1	-1.4	2.1	1	PIR	Pirin	NM_003662
P01112_H01	1.8	-1.6	1.4	1.3	-1.4	-1	PIST	PDZ/coiled-coil domain binding partner for the rho-family GTPase TC10	NM_020399
P01118_H09	-2.4	2.1	-1	-1.8	1.9	1	PITPNM	phosphatidylinositol transfer protein, membrane-associated	NM_004910
P01110_G02	-1.3	1.6	1.3	-4	2	1	PKIB	protein kinase (cAMP-dependent, catalytic) inhibitor beta	NM_032471
P01146_A11	1.4	-1.5	-1	1.8	-1.9	-1	PLA2G4C	phospholipase A2, group IVC (cytosolic, calcium-independent)	NM_003706
P01124_G10	3	-2.5	1	2.5	-3.2	-1.2	PLA2R1	phospholipase A2 receptor 1, 180kDa	NM_007366

P01070_G08	1.8	-1.7	-1	1.9	-2.3	-1.1	PLAU	plasminogen activator, urokinase	NM_002658
P01064_F01	-1.8	2.3	1.2	-1.7	1.9	1.2	PLCL1	phospholipase C-like 1	NM_006226
P01118_E04	2.4	-1.8	1.3	2.3	-1.9	1.1	PLEK2	pleckstrin 2	NM_016445
P01072_A03	5.2	-5.1	1.3	2.1	-1.6	1.2	PLN	phospholamban	NM_002667
P01084_A08	2.8	-2.2	1.1	1.9	-1.8	1.1	PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase) 2	NM_000935
P01063_E04	1.6	-1.7	-1.2	2.4	-1.6	1.1	PLP2	proteolipid protein 2 (colonic epithelium-enriched)	NM_002668
P01130_B04	-3.6	4.3	1	-5.6	5.1	1.1	PMP2	peripheral myelin protein 2	NM_002677
P01131_C08	-2.2	1.6	1.1	-3.4	2.3	1.1	PNUTL2	peanut-like 2 (Drosophila)	NM_004574
P01106_F02	1.5	-1.3	1.2	1.8	-1.7	1.1	PODXL	podocalyxin-like	NM_005397
P01074_B08	2.8	-1.8	1.2	2.2	-2.8	1.1	POLD3	polymerase (DNA directed), delta 3	BC020587
P01080_A04	-1.3	1.4	1	-1.8	1.5	1.1	PP	pyrophosphatase (inorganic)	NM_021129
P01123_E01	-2.9	3.2	1.3	-3.1	2.8	1.3	PPAP2B	phosphatidic acid phosphatase type 2B	NM_003713
P01064_B12	-1.5	1.7	1.2	-2.2	1.5	-1	PPARG	peroxisome proliferative activated receptor, gamma	NM_005037
P01136_D03	-5.4	3.3	1.1	-5.3	4.2	-1	PPL	periplakin	NM_002705
P01131_H04	1.2	-1.4	-1.2	2	-1.6	-1.2	PPP2R4	protein phosphatase 2A, regulatory subunit B' (PR 53)	NM_021131
P01087_D04	-1.2	1.3	-1.1	-1.9	1.5	-1.1	PRKCM	protein kinase C, mu	NM_002742
P01128_H07	2.3	-2.2	1.3	1.4	-1.4	1.1	PRPS1	phosphoribosyl pyrophosphate synthetase 1	NM_002764
P01062_F06	-1.6	1.5	1.1	-4.4	3.6	1	PSG1	pregnancy specific beta-1-glycoprotein 1	NM_006905
P01133_G04	-2	1.9	1.2	-5.5	4.8	-1	PSG1	pregnancy specific beta-1-glycoprotein 1	NM_006905
P01131_G08	-1.4	1.4	1.2	-2.6	2.6	1.1	PSG11	pregnancy specific beta-1-glycoprotein 11	NM_002785
P01141_B07	-1.4	1.8	1.3	-4.1	4	1.1	PSG4	pregnancy specific beta-1-glycoprotein 4	NM_002780
P01079_F07	-1.5	1.5	1.1	-2	1.5	-1	PTGER4	prostaglandin E receptor 4 (subtype EP4)	NM_000958
P01131_C07	-2.8	1.7	-1.1	-2.2	1.8	-1.1	PTGIS	prostaglandin I2 (prostacyclin) synthase	NM_000961
P01102_D10	2.3	-2.8	-1.1	1.1	-1.2	-1.1	PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	NM_000962
P01087_D05	3	-2.6	1.1	1.3	-1.2	-1.1	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	NM_000963
P01106_G06	1.8	-1.5	1.2	3.1	-1.5	1.1	PTHLH	parathyroid hormone-like hormone	NM_002820
P01071_G12	-1.9	1.4	-1	-3.7	3.6	-1.1	PTN	pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1)	NM_002825
P01128_H08	-2.3	2.4	1.1	-1.5	1.3	-1	PTTG1	pituitary tumor-transforming 1	NM_004219
P01095_A03	-2.4	2.4	1.2	-1.4	1.2	1	PTTG1	pituitary tumor-transforming 1	NM_004219
P01097_G06	-1.7	1.7	-1	-2.2	1.6	-1	PUS1	pseudouridylate synthase 1	NM_025215
P01076_C04	2.5	-1.7	1.2	3.1	-2.6	1.2	QPCT	glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	NM_012413
P01129_C05	-2.1	1.8	-1	-1.5	2.1	1.2	RAB13	RAB13, member RAS oncogene family	NM_002870
P01115_G01	-1.8	1.5	1.1	-1.6	2.2	1.2	RAB13	RAB13, member RAS oncogene family	NM_002870
P01110_E09	1.4	-1.2	1.2	1.8	-1.6	1	RAI	RelA-associated inhibitor	NM_006663
P01100_E02	-1.5	1.5	1.1	-3.3	2.7	1	RAI3	retinoic acid induced 3	NM_003979

P01082_A01	-2.4	1.8	1.1	-2.6	2.3	1.1	RARRES3	retinoic acid receptor responder (tazarotene induced) 3	NM_004585
P01117_H10	-1.8	1.6	1.1	-2.5	2	1	RASSF5	Ras association (RalGDS/AF-6) domain family 5	NM_031437
P01108_C07	1.4	-1.4	1.1	2.5	-1.9	1.2	RBP1	retinol binding protein 1, cellular	NM_002899
P01136_C04	2.6	-1.8	1.1	2.3	-1.6	1.2	RGS2	regulator of G-protein signalling 2, 24kDa	NM_002923
P01145_A10	-1.2	1.2	-1.1	-2	1.6	-1.1	RGS4	regulator of G-protein signalling 4	NM_005613
P01090_D02	-1.3	1.2	-1.1	-3	1.9	-1.3	RGS4	regulator of G-protein signalling 4	NM_005613
P01081_H10	-2.2	1.6	1	-6.7	4.7	-1	RGS5	regulator of G-protein signalling 5	NM_003617
P01071_E04	-1.9	1.4	-1.1	-3.8	3.2	-1.1	RNASE1	ribonuclease, RNase A family, 1 (pancreatic)	NM_002933
P01088_G09	-1	1	1	-1.8	1.8	-1.1	RPL5	ribosomal protein L5	NM_000969
P01127_E10	1.8	-1.6	1.1	1.7	-1.5	-1	RRAS	related RAS viral (r-ras) oncogene homolog	NM_006270
P01122_B03	-2	2	1.1	-2.4	3.1	1.2	RRP4	homolog of Yeast RRP4 (ribosomal RNA processing 4), 3'-5'-exoribonuclease	NM_014285
P01104_D09	2.1	-1.7	1.1	2	-1.8	1	RTP801	HIF-1 responsive RTP801	NM_019058
P01121_G04	2.1	-1.8	1.1	4.1	-3.2	1.1	RUVBL2	RuvB-like 2 (E. coli)	NM_006666
P01087_B06	-1.4	1	-1.2	-1.9	2.4	-1.2	S100A10	S100 calcium binding protein A10 (annexin II ligand, calpactin I, light polypeptide (p11))	NM_002966
P01064_F10	1.5	-1.5	-1.3	1.8	-1.5	-1.2	S100A11	S100 calcium binding protein A11 (calgizzarin)	NM_005620
P00777_A05	-1.9	1.7	1.1	-2.3	2.4	1.1	S100A4	S100 calcium binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)	NM_002961
P00777_A06	-1.9	1.8	1.1	-2.6	2.7	1.1	S100A4	S100 calcium binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)	NM_002961
P01143_A11	-1.7	1.7	1.1	-2.4	2.4	1.1	S100A4	S100 calcium binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)	NM_002961
P01141_F03	1.5	-1.2	1.3	3.9	-1.7	1.3	SAA2	serum amyloid A2	NM_030754
P01061_F04	-3.1	4	1.3	-2.2	2.8	1.3	SAT	spermidine/spermine N1-acetyltransferase	NM_002970
P01124_B03	-2.9	3.7	1.4	-2.1	2.5	1.4	SAT	spermidine/spermine N1-acetyltransferase	NM_002970
P01140_G05	2	-2.1	1.1	1.3	-1.3	-1	SC5DL	sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, fungal)-like	NM_006918
P01066_H04	4.1	-2.7	1.2	3	-2	1.2	SCD	stearoyl-CoA desaturase (delta-9-desaturase)	NM_005063
P01140_D11	4.7	-3.8	1.2	3.5	-2.4	1	SCD	stearoyl-CoA desaturase (delta-9-desaturase)	NM_005063
P01119_B12	-1.6	1.9	1.2	-3.9	2.3	1.1	SCDGF-B	spinal cord-derived growth factor-B	NM_025208
P01087_A04	-1.1	1.3	1.2	-4	2	1.2	SCG2	secretogranin II (chromogranin C)	NM_003469
P01096_B12	2.6	-1.9	1.2	2.8	-2.5	-1	SCRG1	scrapie responsive protein 1	NM_007281
P01071_B04	-1.7	1.7	-1.1	-2.6	2.3	1	SDC4	syndecan 4 (amphiglycan, ryudocan)	NM_002999
P01063_H09	-1.8	1.7	1	-1.8	1.6	-1	SDCBP	syndecan binding protein (syntenin)	NM_005625

P01076_C05	1.8	-1.5	1.2	1	-1.2	-1	SEC23A	Sec23 homolog A (S. cerevisiae)	NM_006364
P01096_G04	-3.6	2.5	-1	-3	6	1.3	SELENBP1	selenium binding protein 1	NM_003944
P01119_G09	-3.2	2.4	1.1	-2.5	5.8	1.4	SELENBP1	selenium binding protein 1	NM_003944
P01076_B03	-1.6	1.4	-1	-2	1.5	-1.1	SEPP1	selenoprotein P, plasma, 1	NM_005410
P01062_D11	3	-2.9	-1	4.3	-3.3	-1	SERPINE1	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	NM_000602
P01090_H11	-1.3	1.2	1.2	-1.9	2.1	1.3	SFRP1	secreted frizzled-related protein 1	NM_003012
P01078_F01	-1.8	2.4	-1.1	-1.6	1.6	1.1	SFRP4	secreted frizzled-related protein 4	NM_003014
P01087_A06	-2.9	1.9	-1.2	-3	2.2	-1.3	SGNE1	secretory granule, neuroendocrine protein 1 (7B2 protein)	NM_003020
P01106_G05	1.8	-1.7	1.3	2.9	-2.2	1.2	SKIL	SKI-like	NM_005414
P01102_A06	-1.8	2	1.3	-3.2	2.8	1.3	SLC11A3	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 3	NM_014585
P01105_A03	1.9	-1.7	1.1	1.5	-1.4	-1	SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	NM_003038
P01143_D11	-2.7	2.5	1.3	-2.1	2.8	1.1	SLC25A11	solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11	NM_003562
P01111_H03	1.8	-1.7	1	1.9	-2	-1	SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	NM_014331
P01138_A08	3	-2.9	-1	2.3	-2.4	-1.1	SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	NM_003486
P01088_E10	3.1	-2.7	1.1	2.6	-2.3	1	SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	NM_003486
P01112_E05	-1.6	1.3	1	-3	2.2	-1	SLIT3	slit homolog 3 (Drosophila)	NM_003062
P01136_F07	-1.1	1.4	1.2	-2.1	1.9	1.1	SLIT3	slit homolog 3 (Drosophila)	NM_003062
P01079_G03	-3.1	3.4	-1	-3.9	3.5	-1	SNAI2	snail homolog 2 (Drosophila)	NM_003068
P01140_F07	2.9	-2.6	1.1	2.2	-2.5	1.1	SNF1LK	SNF1-like kinase	
P01083_A04	-3.2	3.2	1	-9.3	7	-1.1	SNK	serum-inducible kinase	NM_006622
P01085_F06	-1.2	1.2	1.1	-2.6	1.7	1.1	SOD3	superoxide dismutase 3, extracellular	NM_003102
P01074_H12	1	1.1	1.1	-2.6	1.5	1.1	SPINT2	serine protease inhibitor, Kunitz type, 2	NM_021102
P01108_B02	-2.5	2.6	1.2	-4.2	2.2	-1	SPRY1	sprouty homolog 1, antagonist of FGF signaling (Drosophila)	AF041037
P01095_F04	-2.6	2	-1.1	-1.8	1.8	-1.1	SQRDL	sulfide quinone reductase-like (yeast)	NM_021199
P01128_E07	1.9	-2	1	2.6	-2.7	-1	SRPUL	sushi-repeat protein	NM_014467
P01073_B02	-1.7	1.7	1.2	-2.5	1.9	-1	SRPX	sushi-repeat-containing protein, X chromosome	NM_006307
P01104_F12	-2.1	2.5	1.2	-2.2	2.3	-1.1	SSBP2	single-stranded DNA binding protein 2	NM_012446
P01069_C06	1.9	-1.3	-1	2.7	-2.6	-1	SSR1	signal sequence receptor, alpha (translocon-associated protein alpha)	NM_003144
P01130_F10	-1.3	1.6	1.1	-2.3	2.3	-1.1	STC1	stanniocalcin 1	NM_003155
P01130_B11	2.1	-2	-1	1.7	-1.8	-1.1	STCH	stress 70 protein chaperone, microsome-associated, 60kDa	NM_006948
P01074_E03	1.7	-1.3	-1	-1.9	1.5	-1.1	STE	sulfotransferase, estrogen-	NM_005420

								preferring	
P01127_G01	-1.4	1.5	1.2	-1.9	1.5	1.2	STK17B	serine/threonine kinase 17b (apoptosis-inducing)	NM_004226
P01125_C11	-2	1.6	-1	-2.2	2.1	-1	STK25	serine/threonine kinase 25 (STE20 homolog, yeast)	NM_006374
P01076_D03	-2.7	2.9	1.1	-2.1	1.8	1	STK38	serine/threonine kinase 38	NM_007271
P01105_E03	-2.8	2.7	-1.1	-2.7	2.5	-1.1	STMN1	stathmin 1/oncoprotein 18	NM_005563
P01069_A08	-1.5	1.5	1.1	-1.8	1.5	1	STOM	stomatin	NM_004099
P01102_E10	-1.5	1.6	1.1	-2.3	1.5	1	SVIL	supervillin	NM_003174
P01062_H06	-1.5	2.1	1.2	-2.9	2.7	1.2	TACSTD2	tumor-associated calcium signal transducer 2	NM_002353
P01098_E05	1.9	-1.8	1.2	1.2	-1.3	1.1	TAF13	TAF13 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 18kDa	NM_005645
P01101_B02	-1.9	1.4	1.1	-2	1.8	1	TCF7L1	transcription factor 7-like 1 (T-cell specific, HMG-box)	NM_031283
P01061_C01	-1.7	1.6	1.3	-2	2.4	1.1	TF	transferrin	NM_001063
P01144_C03	-3.4	3.6	1.2	-4	2.9	1.1	TFPI	tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	NM_006287
P01071_A04	-1.4	1.6	1.4	-2.3	2.3	1.1	TFPI2	tissue factor pathway inhibitor 2	NM_006528
P01085_B12	-1.4	1.4	1.2	-2.1	1.7	1.1	TGFB2	transforming growth factor, beta 2	NM_003238
P01061_C08	-3.5	3.8	1.2	-4.7	4	1.2	TGFBR3	transforming growth factor, beta receptor III (betaglycan, 300kDa)	NM_003243
P01078_B04	1.8	-1.7	-1	1.8	-1.9	-1.2	THBS2	thrombospondin 2	NM_003247
P01124_G04	2.8	-2.5	-1	2.4	-3.1	-1.3	TIMP3	tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	NM_000362
P01086_F06	2.1	-2.4	-1	2.6	-3.1	-1.2	TIMP3	tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	NM_000362
P01071_A06	-3	3	-1	-2.5	2.6	-1	TM4SF1	transmembrane 4 superfamily member 1	NM_014220
P01099_E08	-1.6	1.8	1	-1.8	1.5	-1.1	TncRNA	trophoblast-derived noncoding RNA	
P01126_E09	-1.7	2	1	-3.7	4.2	1.1	TNFAIP2	tumor necrosis factor, alpha-induced protein 2	NM_006291
P01085_A06	-1.5	1.6	-1	-2.4	1.9	1.1	TNFAIP3	tumor necrosis factor, alpha-induced protein 3	NM_006290
P01138_G10	1.8	-1.7	1.2	2.1	-2	1	TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A	NM_016639
P01078_E05	-2.1	3	1.4	-2.6	2.5	1.2	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	NM_003810
P01144_C11	-2	2	1	-1.9	1.4	1.2	TOP2A	topoisomerase (DNA) II alpha 170kDa	NM_001067
P01140_D03	2.1	-1.7	-1	1.2	-1.6	-1	TTID	titin immunoglobulin domain protein (myotilin)	NM_006790
P01070_H07	-2.9	2.3	1.1	-3	2.6	-1.1	TXNRD1	thioredoxin reductase 1	NM_003330
P01089_D01	1.7	-3	1.1	2.5	-2.3	-1	UCHL1	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	NM_004181
P01123_D07	-1.9	2.4	1.1	-2	1.8	1.1	UGCG	UDP-glucose ceramide glucosyltransferase	NM_003358
P01089_F07	1.5	-2.6	1.2	2.4	-1.7	1.2	UMPK	uridine monophosphate kinase	NM_012474

P01070_F11	2.1	-3.1	1.1	2.4	-1.9	1	UMPS	uridine monophosphate synthetase (orotate phosphoribosyl transferase and orotidine-5'-decarboxylase)	NM_000373
P01061_B02	-2.7	2.4	1	-4.2	2.3	-1.3	VCAM1	vascular cell adhesion molecule 1	NM_001078
P01141_C06	2.7	-1.8	1.3	1.4	-1.2	1.2	WISP1	WNT1 inducible signaling pathway protein 1	NM_003882
P00777_C09	-1.6	1.8	1.1	-5	4	-1	WISP2	WNT1 inducible signaling pathway protein 2	NM_003881
P00777_C10	-2.2	2.1	1.1	-5.6	4.4	-1	WISP2	WNT1 inducible signaling pathway protein 2	NM_003881
P01126_H07	-1.8	1.6	1.1	-3	3.9	-1	WISP2	WNT1 inducible signaling pathway protein 2	NM_003881
P01142_D08	3.7	-3	1.3	3.6	-2.8	1.1	XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4	NM_003401
P01104_H07	-1.7	1.7	1.2	-1.9	1.5	1.1	ZFPM2	zinc finger protein, multitype 2	NM_012082
P01064_H12	-1.4	1.5	1.1	-1.8	1.5	-1	ZNF142	zinc finger protein 142 (clone pHZ-49)	NM_005081
P01075_E02	1.5	-1.2	1.1	1.9	-1.9	1	ZNF193	zinc finger protein 193	NM_006299

Validation of Microarray by Real-time RT-PCR and Western Blot Analyses

Representative microarray data was validated using real-time RT-PCR and Western analyses. TGFβ induced *Collagen 1* mRNA levels in human cardiac fibroblasts at 6, 24, and 48 h; this induction was blocked by BNP at all 3 time points (Fig. 5A). Collagen 1 protein synthesis was also induced (~3-fold) at 48 h, and BNP inhibited this stimulation by ~75% (Fig. 5B). BNP also inhibited TGFβ-induced *Fibronectin* mRNA and protein expression at 48 h (Fig. 5C,D). These data corroborate the microarray results, with the exception of *Fibronectin,* which did not exceed the array differential expression threshold value, most likely due to the lower sensitivity of the microarray compared to real-time RT-PCR. The effects of BNP on TGFβ stimulation of pro-fibrotic genes *CTGF, PAI-1, TIMP3, IL11,* and *ACTA2* were also confirmed by real-time RT-PCR (Fig. 6). Additional verification was obtained for the pro-inflammatory genes *COX2* and *IL6* at 6, 24, and 48 h (Fig. 6). Again, most likely due

to sensitivity issues, *IL6* was not included in Fig. 4C, since it did not exceed the array differential expression threshold value.

In addition, real-time RT-PCR assays were performed for 9 genes on primary cultures of human cardiac fibroblasts from a second independent donor lot of fibroblasts (see Table 3). The effects of BNP on TGFβ-induced gene expression in both donors were similar, although donor lot 2 was slightly less responsive to TGFβ. Taken together, these results confirm the microarray data using independent assay methods, as well as, multiple human cardiac fibroblast donors.

Table 3. Real-time RT-PCR validation of microarray data using human cardiac fibroblasts from two separate donors (lot 1 and lot 2). Expression levels are normalized to 18s RNA and are shown relative to the control samples. Standard deviations reflect duplicate biological replicates; real-time RT-PCR reactions were performed in triplicate.

Gene	Control	BNP	TGFβ	TGFβ +BNP	Time (h)	Lot
Collagen 1	1.0±0.05	1.0±0.05	1.9±0.04	1.2±0.01	6	1
	1.0±0.06	1.1±0.13	3.3±0.05	1.3±0.26	24	1
	1.0±0.11	1.0±0.26	1.5±0.09	1.2±0.01	24	2
	1.0±0.13	1.2±0.03	3.8±0.38	1.3±0.03	48	1
	1.0±0.20	1.0±0.01	2.5±0.32	1.3±0.18	48	2
Fibronectin	1.0±0.04	0.9±0.19	1.1±0.17	1.0±0.29	6	1
	1.0±0.21	1.0±0.10	1.0±0.05	1.0±0.18	24	1
	1.0±0.19	0.9±0.24	1.0±0.02	1.0±0.12	24	2
	1.0±0.04	1.1±0.04	2.2±0.38	1.3±0.35	48	1
	1.0±0.01	1.0±0.11	2.0±0.39	1.5±0.02	48	2
SERPINE1/PAI-1	1.0±0.07	0.7±0.08	7.3±0.44	1.7±0.37	6	1
	1.0±0.01	0.7±0.01	8.5±0.08	0.7±0.10	24	1
	1.0±0.10	0.7±0.11	2.4±0.06	1.1±0.10	24	2
	1.0±0.22	0.9±0.00	8.4±1.33	0.9±0.13	48	1
	1.0±0.17	0.8±0.03	2.6±0.03	0.9±0.06	48	2
CTGF	1.0±0.15	0.9±0.24	3.5±0.08	0.9±0.03	6	1
	1.0±0.28	1.0±0.29	3.3±0.25	0.7±0.25	24	1
	1.0±0.09	1.5±0.44	2.2±0.16	1.5±0.04	24	2
	1.0±0.45	1.4±0.13	3.1±0.01	1.1±0.01	48	1
	1.0±0.32	1.3±0.12	2.1±0.14	1.0±0.24	48	2
IL11	1.0±0.20	1.1±0.04	13.3±0.89	2.1±0.06	6	1

	1.0±0.13	1.2±0.07	32.3±0.82	1.1±0.14	24	1
	1.0±0.06	1.0±0.05	7.7±0.81	2.1±0.18	24	2
	1.0±0.23	0.7±0.10	17.6±0.22	1.0±0.08	48	1
	1.0±0.09	0.8±0.09	5.9±0.18	1.2±0.10	48	2
TIMP3	1.0±0.01	0.9±0.11	1.4±0.03	1.0±0.12	6	1
	1.0±0.31	1.0±0.12	2.6±0.26	1.0±0.23	24	1
	1.0±0.13	0.7±0.09	1.5±0.12	1.3±0.14	24	2
	1.0±0.26	0.9±0.00	3.0±0.34	1.0±0.09	48	1
	1.0±0.08	0.6±0.00	1.7±0.13	0.8±0.01	48	2
IL6	1.0±0.06	0.9±0.02	3.6±0.27	1.3±0.14	6	1
	1.0±0.13	0.9±0.21	1.7±0.14	0.8±0.03	24	1
	1.0±0.09	0.9±0.07	1.4±0.05	1.0±0.11	24	2
	1.0±0.13	0.9±0.03	1.6±0.12	0.9±0.05	48	1
	1.0±0.17	0.9±0.06	1.4±0.17	0.9±0.17	48	2
PTGS2/COX-2	1.0±0.01	1.2±0.22	9.0±1.49	1.8±0.05	6	1
	1.0±0.08	1.2±0.38	3.5±0.67	1.2±0.19	24	1
	1.0±0.07	1.1±0.05	4.9±0.36	1.4±0.18	24	2
	1.0±0.10	1.0±0.12	2.2±0.12	1.3±0.03	48	1
	1.0±0.19	1.0±0.06	5.4±0.92	1.2±0.01	48	2
ACTA2	1.0±0.03	0.8±0.12	1.1±0.11	0.9±0.20	6	1
	1.0±0.14	0.9±0.11	2.2±0.00	0.9±0.07	24	1
	1.0±0.04	0.9±0.25	2.3±0.12	1.6±0.41	24	2
	1.0±0.17	1.0±0.03	1.0±0.19	1.0±0.21	48	1
	1.0±0.05	0.7±0.11	2.5±0.13	1.0±0.12	48	2

In a related study, a gene microassay profile of rat heart tissue was conducted. The results of this study are shown in Figure 12. Fibrotic and extracellular matrix associated genes were stimulated in vivo by L-NAME plus angiotensin II. MRNA expression for collagen I, collagen III, and fibronectin was markedly reduced by the administration of BNP.

MEK/ERK pathway involved in BNP's Anti-Fibrotic Role

Natriuretic peptides were previously shown to stimulate ERK activity in cardiac myocytes and vascular endothelial cells. The MEK/ERK pathway has been linked to the repression of TGFβ/Smad signaling. To determine whether PKG or ERK

signaling is involved in BNP-dependent attenuation of TGFβ signaling, cultured cells were treated with BNP and/or TGFβ in the presence of a PKG inhibitor (KT5823) or two different MEK inhibitors (U0126, PD98059). BNP induced ERK phosphorylation was completely blocked by KT5823 and U0126, indicating that BNP activates ERK via PKG and MEK signaling cascades (Fig. 7a). Both MEK inhibitors (U0126, PD98059) reversed BNP inhibition of TGFβ–induced Collagen-1 expression analyzed by Western blot (Fig. 7b) and real-time RT-PCR (Fig. 7c). A similar result was demonstrated for *PAI-1* using real-time RT-PCR. These findings suggest that the ERK pathway plays an important role in BNP-dependent inhibition of the fibrotic response induced by TGFβ in human cardiac fibroblasts.

Fibrosis and ECM

One of the key features of cardiac fibrosis is the increased deposition of the ECM. The dynamic turnover of ECM proteins is controlled by several regulatory mechanisms: de novo biosynthesis of ECM components, proteolytic degradation of ECMs by matrix metalloproteinases (MMPs), and inhibition of MMP activities by endogenous inhibitors, TIMPs. All of these processes have been shown to be profoundly affected by TGFβ. The results provided herein suggest that TGFβ-induced ECM deposition in human cardiac fibroblasts occurs largely by increasing ECM gene expression, including *Fibronectin*, *COL1A2*, *COL15A*, *COL7A1*, *MAGP2, MATN3, FBN1,* and *COMP*. Fibronectin and collagen expression in cardiac fibroblasts has been well-established in the fibrotic response, however, this is the first report of TGFβ induction of other ECM genes including *MAGP2, MATN3, FBN1* and *COMP*, further corroborating TGFβ's role in ECM induction. Interestingly, *COMP*, which is a member of the thrombospondin family, has been shown to have a direct interaction

with Fibronectin,[25] supporting its role in fibrotic processes. We also found *Thombospondin 2,* which is involved in the activation of latent TGFβ[26] regulated by TGFβ in our studies and opposed by BNP (Table 2). Also sharing close identity with the latent TGFβ family of binding proteins is *FBN1*, a component of extracellular microfibrils. The opposing effects of BNP on these gene regulatory events, suggests that BNP modulates cardiac fibrosis.

In addition to the suppression of TGFβ–induced ECM biosynthesis, BNP may also modulate the degradation of ECM proteins by opposing elevated *TIMP3* levels in TGFβ–stimulated cells. The TIMP family of proteins is believed to play significant roles in controlling extracellular matrix remodeling. Elevation of *TIMP3* expression has been observed in animal models of myocardial infarction, suggesting that it may be a contributor to matrix remodeling in the failing heart.

Another hallmark of the fibrotic process is the transformation of cardiac fibroblasts to myofibroblasts and the induction of pro-fibrotic mediators. Myofibroblasts acquire contractile properties similar to smooth muscle cells. The results provided above demonstrate that BNP inhibited TGFβ-induction of several myofibroblast markers including *ACTA2* and *MYH9*. BNP also inhibited TGFβ pro-fibrotic mediators, such as, *CTGF, PAI-1, and IL11*. *CTGF* and *PAI-1* are well-established downstream signaling genes of the TGFβ pathway, and IL11 has been associated with tissue remodeling and fibrosis. IL11 expression in cardiac fibroblasts also seems to contribute to TGFβ-mediated fibrosis. The use of BNP to suppress this response should result in a protective effect.

Collectively, these effects of BNP on gene expression in TGFβ–stimulated cells demonstrate a role for BNP in anti-fibrotic processes in cardiac fibroblasts. In striking contrast to TGFβ-treated cells, BNP had no significant effects in unstimulated

fibroblasts. This is consistent with the physiological actions of BNP, working only in opposition to other hormonal systems such as the renin-angiotensin-aldosterone system.

Changes in Cell Proliferation

The effects of TGFβ on cell growth is cell-type dependent. As provided above, TGFβ stimulated cardiac fibroblast proliferation. Whether TGFβ has a direct effect on cell cycle or an indirect effect through other mechanisms is unclear. However, cDNA microarray analysis revealed that BNP markedly inhibits the expression of a number of TGFβ-induced growth factors or growth factor-like genes including *PDGFA, IGF1, FGF18,* and *IGFBP10* (*CYR61*). The up-regulation of these genes by TGFβ could partially explain the induction of cell proliferation, suggesting that it may be mediated indirectly through the stimulation of growth factor productions. TGFβ also induced the expression of *PTHLH (PTHrP)*, which has known chronotropic and vasodilatory effects. In osteoblast-like cells PTHrP can induce cell proliferation. Interestingly, in the myocardium, PTHrP levels are increased in congestive heart failure (CHF).

The growth inhibitory effects of natriuretic peptides have previously been reported. Cao and Gardner first demonstrated that natriuretic peptides inhibit PDGF, FGF2, and mechanical stretch-induced DNA synthesis in neonatal rat cardiac fibroblasts. Consistent with these findings, natriuretic peptides and cyclic GMP have been reported to inhibit cell proliferation induced by angiontensin II, endothelin-1, and norepinephrine in many cell types including cardiac fibroblasts, vascular smooth muscle cells, endothelial cells, and mesangial cells. The results provided herein

suggest an important role for BNP in regulating fibroblast growth during cardiac remodeling.

Changes in Inflammatory Genes

Cardiac expression of cytokines is thought to contribute to a decrease in left ventricle contractile performance and deleterious remodeling. Although similar effects have been observed with ANP, reported herein for the first time is that *brain natriuretic peptide* blocks TGFβ stimulation of several pro-inflammatory genes including *COX2, IL6, TNFAIP6,* and *TNFSF4.*

TGFβ has a dual effect in the regulation of inflammatory processes. For example, it increases COX2 expression and prostaglandin E2 release in pulmonary artery smooth muscle cells, airway smooth muscle cells, and intestinal epithelial cells. On the other hand, TGFβ down-regulates the production of MCP-1 and complement components (C3 and C4) in human proximal tubular epithelial cells and macrophages. The results provided herein corroborates the dual effect of TGFβ in the modulation of inflammatory gene expression in cardiac fibroblasts. From these results, it was found that while TGFβ induced some inflammatory genes, it down-regulated others, such as, *IL1b, MCP1-R, GRO1, GRO3,* and *MCP4.* Both effects are reversed by BNP. However, in the absence of TGFβ stimulation, BNP had no significant effect on the expression of inflammatory genes. It is likely that a balance of pro- and anti-inflammatory stimuli is important in the process of cardiac remodeling.

Signaling Mechanism Underlying BNP's Anti-Fibrotic Role

Studies aimed at elucidating the mechanism of BNP's inhibition of a fibrotic response indicate that the ERK signaling pathway plays an important role. The results

provided herein demonstrate that BNP phosphorylates ERK via PKG-dependent signaling in primary human cardiac fibroblasts. Moreover, this activation attenuates the TGFβ-induced fibrotic response as measured by Collagen 1 expression. This is consistent with previous studies showing that ERK activation is required for both the anti-hypertrophic effect of ANP in cardiac myocytes, and the inhibition of TGFβ signaling in mammary and lung epithelial cells.

In vivo studies

In a related study, an *in vivo* model for acute myocardial injury was used to explore the effects of BNP. Male Sprague Dawley rats ranging in weight from 225 to 250 gm were utilized. Acute myocardial injury was induced by administration of *N*ω-nitro-L-arginine methyl ester (L-NAME, 40 mg/kg/day)salt (1%NaCl) plus angiotensin II (AngII, 0.5 mg/kg/day) in the rats. The L-NAME was administered in drinking water from day 1 to day 14. Angiotensin II was continuously infused subcutaneously with an osmotic pump from day 11 to day 14. Rat BNP (400 mg/kg/min) was intravenously infused through an external infusion pump from day 10 to day 14.

Systolic blood pressure, plasma level of aldosterone, cardiac function heart/body weight ration and gene expression in the heart were analyzed. Systolic blood pressure was monitored via tail cuff technique with an IITC blood pressure recording system. Cardiac function was monitored via a Millar ARIA Pressure Volume Conductance System with an 1.4 F catheter. Gene expression as referenced above with results provided in Figure 12 were monitored by RT-PCR with an ABI Prism TM 7700 sequence detection system.

It was observed that BNP had no effect on systolic blood pressure raised by L-NAME+AngII but significantly attenuated aldosterone(1.25.2±0.2 vs. 6.6±0.16 ng/ml,

$p<0.05$). See Figure 10. As shown in Figure 13, BNP improved cardiac function by significantly increase in stroke volume (2.68 ± 0.23 vs. 4.74 ± 0.73 ul, $p<0.05$), ejection fraction (13.6 ± 1.1 vs. $20.4\pm2.4\%$ $p<0.05$), and diastolic volume (19.0 ± 0.9 vs 22.4 ± 1.1ul, $p<0.05$) and stroke work (223.0 ± 29.4 vs 531.5 ± 99.1mmH*ul,$p<0.05$), and decrease in arterial elastance (6.50 ± 5.7 vs 42.6 ± 5.1 mmHg/ul, $p<0.01$). As shown in Figure 11, BNP significantly reduced the heart/body weigh ratio (0.0039 ± 0.002 vs. 0.0029 ± 0.001, $p<o.05$) and as referenced above, abolished the profibrotic phenotype indicated by decreasing expression of collagen I ($p<0.01$), collagen III ($p<0.05$) and fibronectin ($p<0.05$).

Summary

Along with the endothelin pathway, the renin-angiotensin and aldosterone system, the fibrosis-promoting TGFβ pathwayis important in the pathophysiology of heart failure. BNP appears to oppose TGFβ-regulated gene expression related to fibrosis and myofibroblast conversion. Furthermore, BNP's opposition to the TGFβ-stimulated fibrotic response is dependent on the PKG and the MEK/ERK pathways. This finding is consistent with the observation that BNP deficient mice show increased fibrosis and *Collagen 1* expression. In addition to BNP's global effects on fibrosis, it may also have effects on other processes, such as inflammation and proliferation (Fig. 8). These findings support a beneficial role for BNP in the prevention of cardiac fibrosis and the treatment of cardiac diseases. They also provide the first demonstration that BNP has a direct effect on cardiac fibroblasts to oppose a TGFβ-induced fibrotic response, suggesting that BNP functions as an anti-fibrotic factor in the heart to prevent cardiac remodeling in pathological conditions.

Independent from the antifibrotic effect, the in vivo studies as provided herein indicate that BNP may be used to reduce cardiac remodeling and prevent subsequent heart failure. BNP may also be useful as a cardioprotective agent to improve cardiac function post acute myocardial injury such as myocardial infarction.

All references cited throughout the specification are expressly incorporated herein by reference. While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, and the like. All such modifications are within the scope of the claims appended hereto.

What is claimed is:

1. A method for treating cardiac remodeling in a subject that has undergone myocardial injury, said method comprising administering a therapeutically effective amount of natriuretic peptide to said subject.

2. A method for treating cardiac dysfunction in a subject that has undergone myocardial injury, said method comprising administering a therapeutically effective amount of natriuretic peptide to said subject.

3. A method for treating cardiac fibrosis in a subject who has undergone myocardial injury, said method comprising administering a therapeutically effective amount of natriuretic peptide to said subject.

4. The method of claims 1 or 2 wherein said natriuretic peptide is brain natriuretic peptide.

5. A method of inhibiting the production of Collagen 1, Collagen 3 or Fibronectin protein in a subject who has undergone myocardial injury, said method comprising administering a therapeutically effective amount of brain natriuretic peptide to said subject.

6. A method of alleviating or reversing the effect of TGFβ mediated cell activation in cardiac tissue on the expression of one or more genes associated with fibrosis, comprising contacting one or more cells or tissues in which the expression of said genes is altered as a result of TGFβ mediated activation, with brain natriuretic peptide.

7. The method of claim 5 wherein said genes are selected from the group consisting essentially of Collagen1, Collagen 3, Fibronectin, CTGF, PAI-1, and TIMP3.

8. A method of inhibiting the transformation of cardiac fibroblast cells into myofibroblast cells in a subject that has undergone myocardial injury, said method comprising administering a therapeutically effective amount of brain natriuretic peptide to said subject.

Figure 1

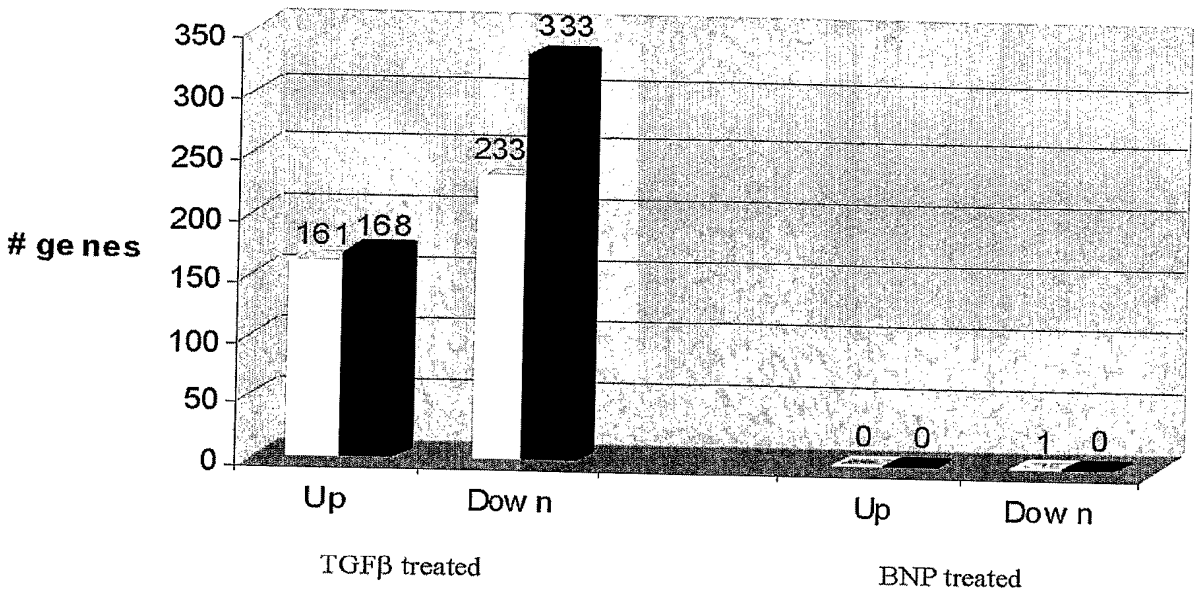

genes
350
300
250
200
150
100
50
0
161
168
233
333
0
0
1
0
Up
Down
Up
Down
TGFβ treated
BNP treated

Figure 2

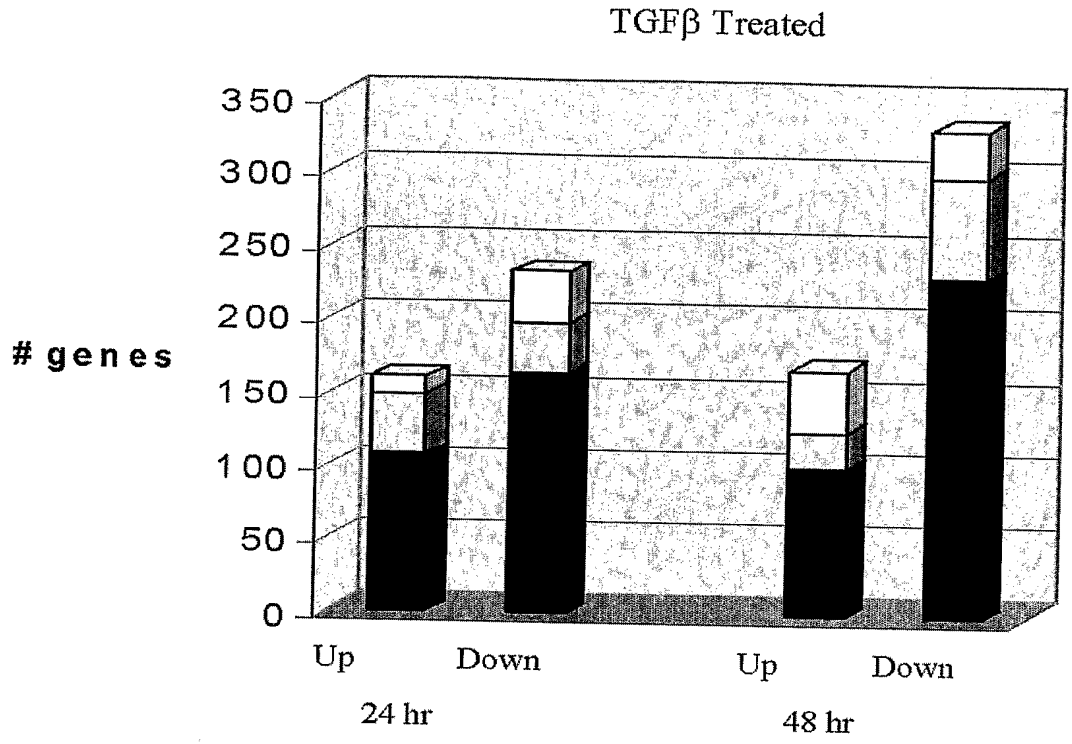

TGFβ Treated
genes
350
300
250
200
150
100
50
0
Up
Down
Up
Down
24 hr
48 hr

Figure 3

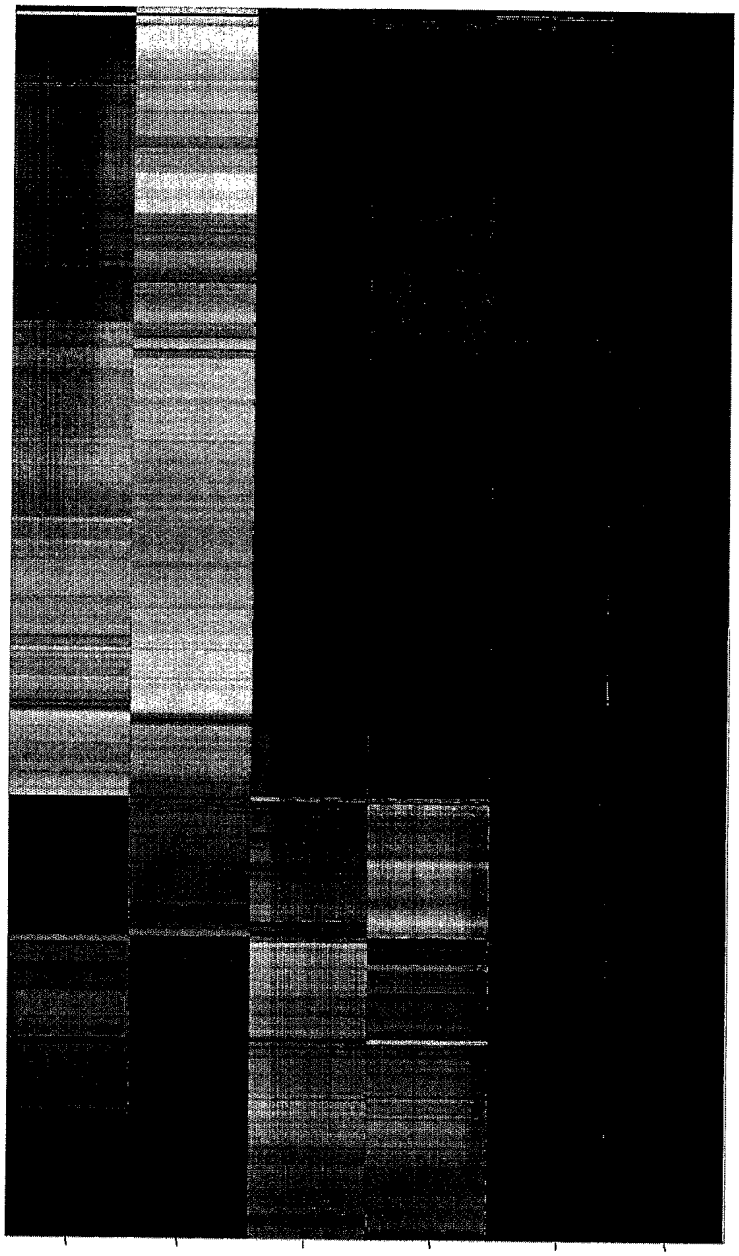

TGFβ
24h
A
TGFβ
48h
B
TGFβ
BNP
24h
C
TGFβ
BNP
48h
D
BNP
24h
E
BNP
48h
F

Figure 4

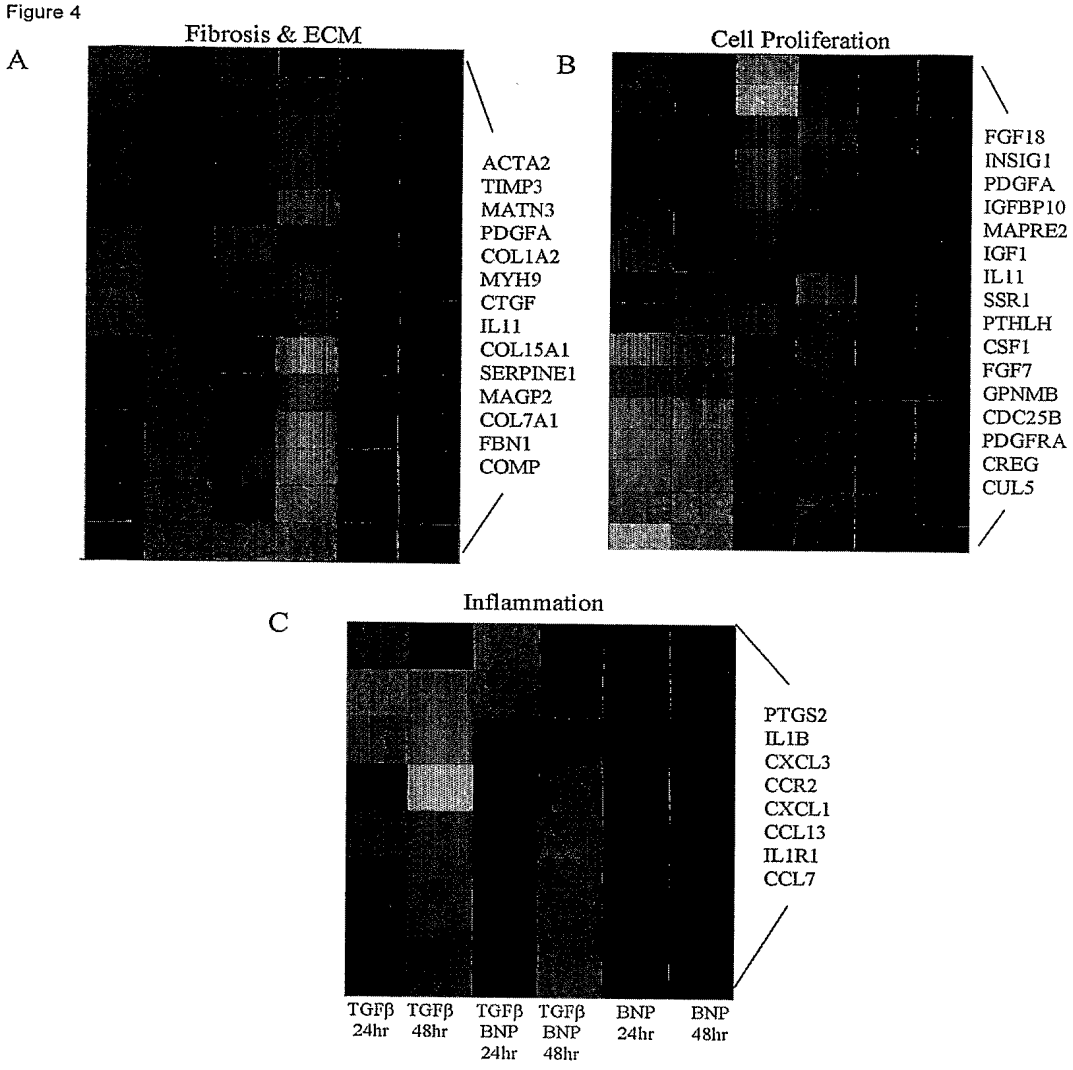

A
Fibrosis & ECM
ACTA2
TIMP3
MATN3
PDGFA
COL1A2
MYH9
CTGF
IL11
COL15A1
SERPINE1
MAGP2
COL7A1
FBN1
COMP
B
Cell Proliferation
FGF18
INSIG1
PDGFA
IGFBP10
MAPRE2
IGF1
IL11
SSR1
PTHLH
CSF1
FGF7
GPNMB
CDC25B
PDGFRA
CREG
CUL5
C
Inflammation
PTGS2
IL1B
CXCL3
CCR2
CXCL1
CCL13
IL1R1
CCL7
TGFβ 24hr
TGFβ 48hr
TGFβ BNP 24hr
TGFβ BNP 48hr
BNP 24hr
BNP 48hr

Figure 5

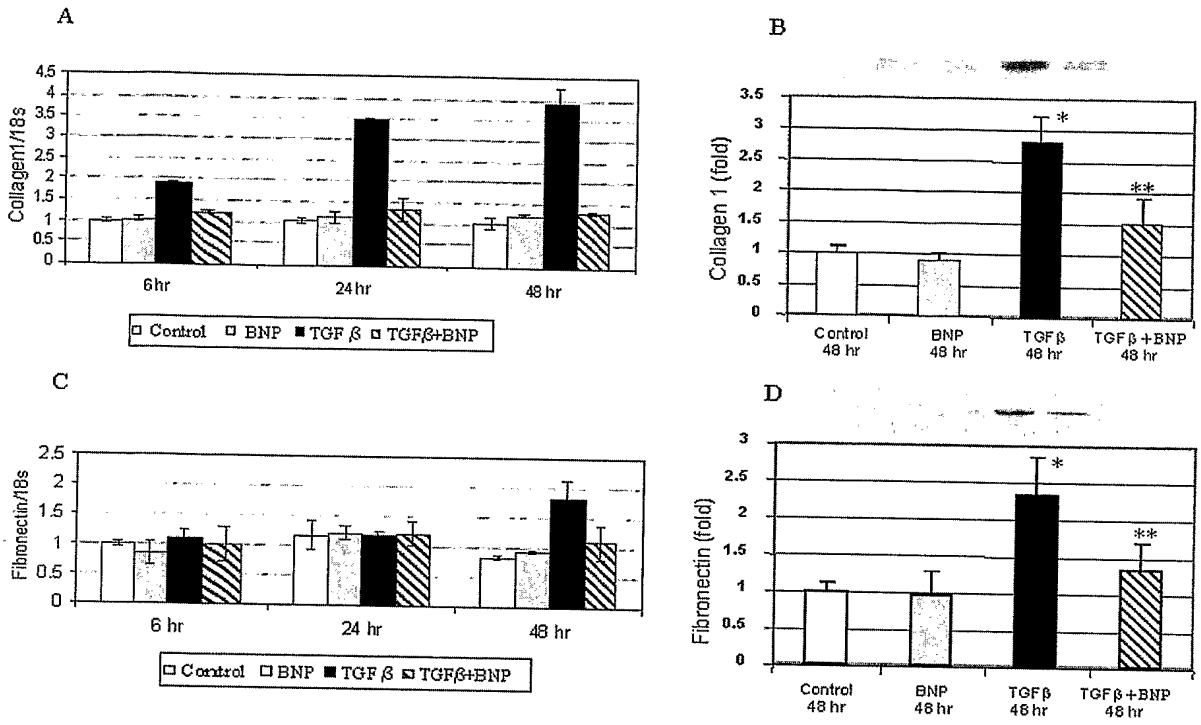

A
Collagen1/18s
6hr
24hr
48 hr
Control
BNP
TGF β
TGFβ+BNP
B
Collagen 1 (fold)
Control 48 hr
BNP 48 hr
TGF β 48 hr
TGF β +BNP 48 hr
C
Fibronectin/18s
6 hr
24 hr
48 hr
Control
BNP
TGF β
TGFβ+BNP
D
Fibronectin (fold)
Control 48 hr
BNP 48 hr
TGF β 48 hr
TGF β +BNP 48 hr

Figure 6

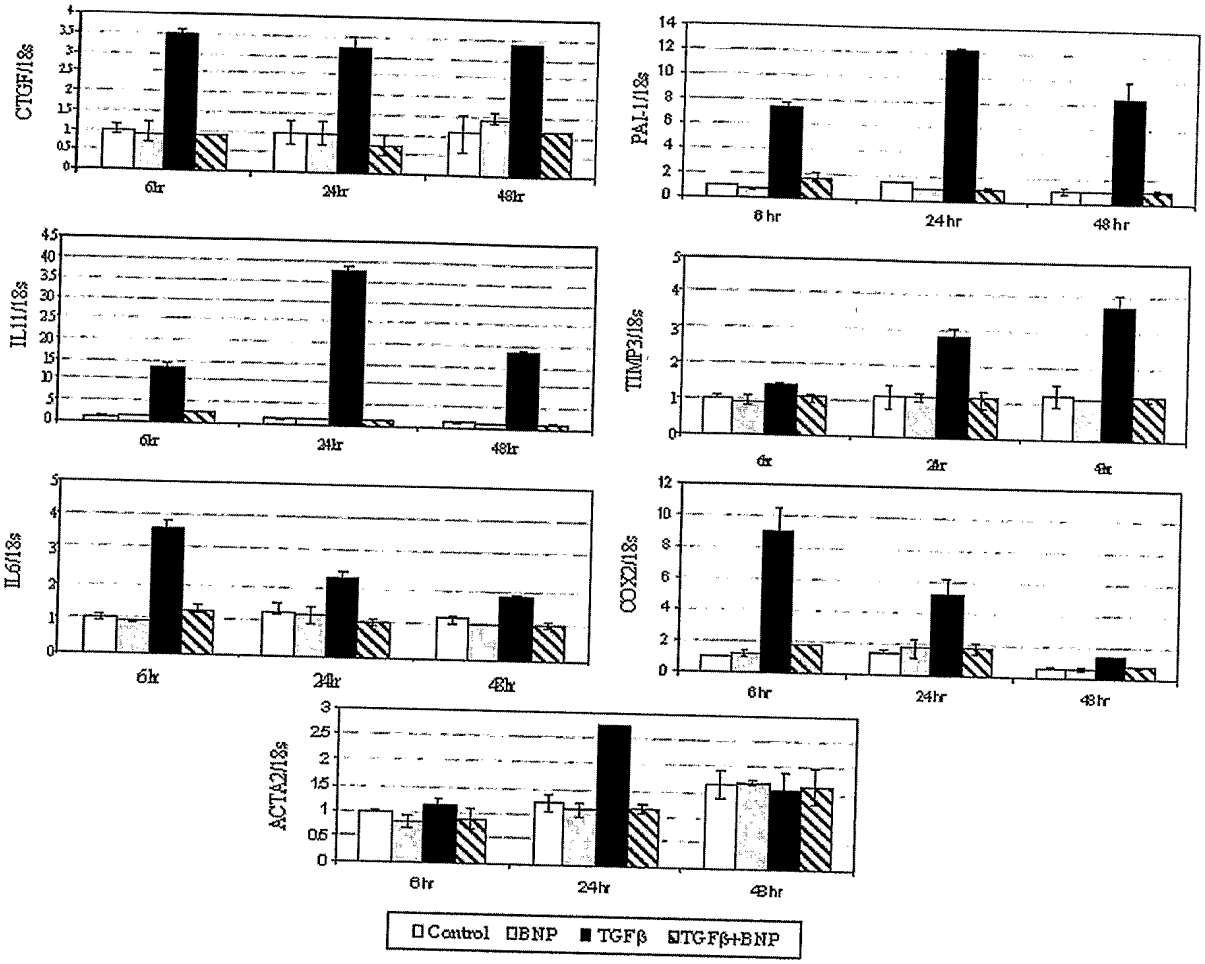
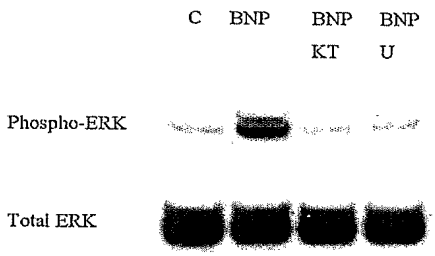
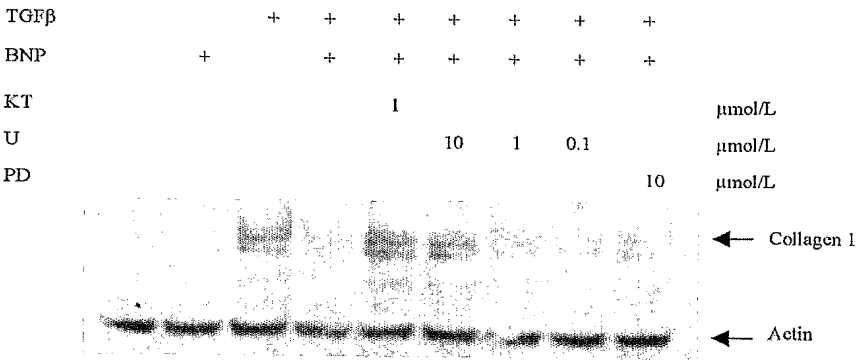

CTGF/18s
PAI-1/18s
IL11/18s
TIMP3/18s
IL6/18s
COX2/18s
ACTA2/18s
6hr
24hr
48hr
Control
BNP
TGFβ
TGFβ+BNP

Figure 7

A


C
BNP
BNP KT
BNP U
Phospho-ERK
Total ERK

B


TGFβ + + + + + + +
BNP + + + + + + +
KT 1 μmol/L
U 10 1 0.1 μmol/L
PD 10 μmol/L
Collagen 1
Actin

C

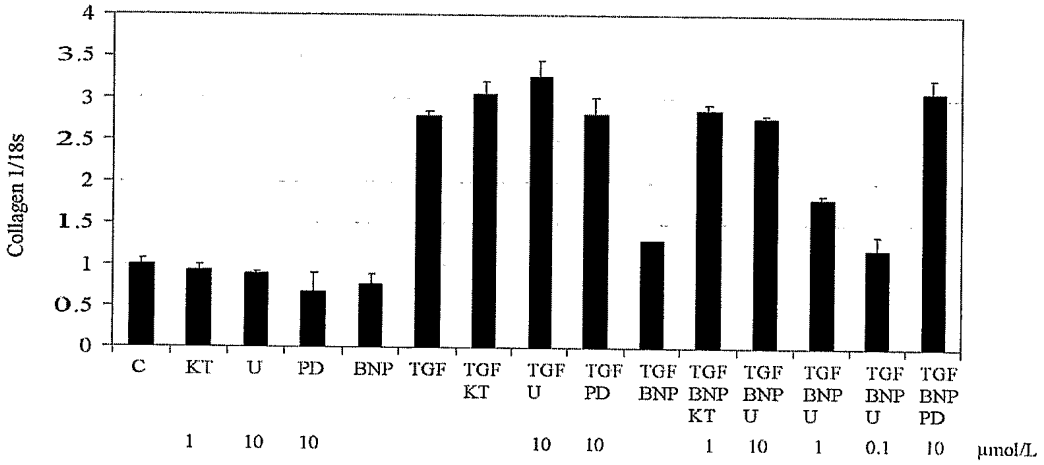

Collagen 1/18s
4
3.5
3
2.5
2
1.5
1
0.5
0
C KT U PD BNP TGF TGF KT TGF U TGF PD TGF BNP TGF BNP KT TGF BNP U TGF BNP U TGF BNP U TGF BNP PD
1 10 10 10 10 1 10 1 0.1 10 μmol/L

Figure 8

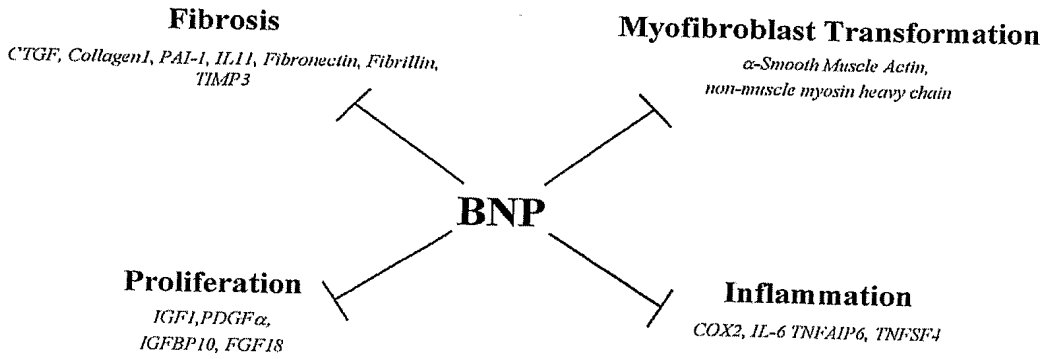

Fibrosis
CTGF, Collagen1, PAI-1, IL11, Fibronectin, Fibrillin, TIMP3
Myofibroblast Transformation
α-Smooth Muscle Actin, non-muscle myosin heavy chain
BNP
Proliferation
IGF1,PDGFα, IGFBP10, FGF18
Inflammation
COX2, IL-6 TNFAIP6, TNFSF4

Figure 9

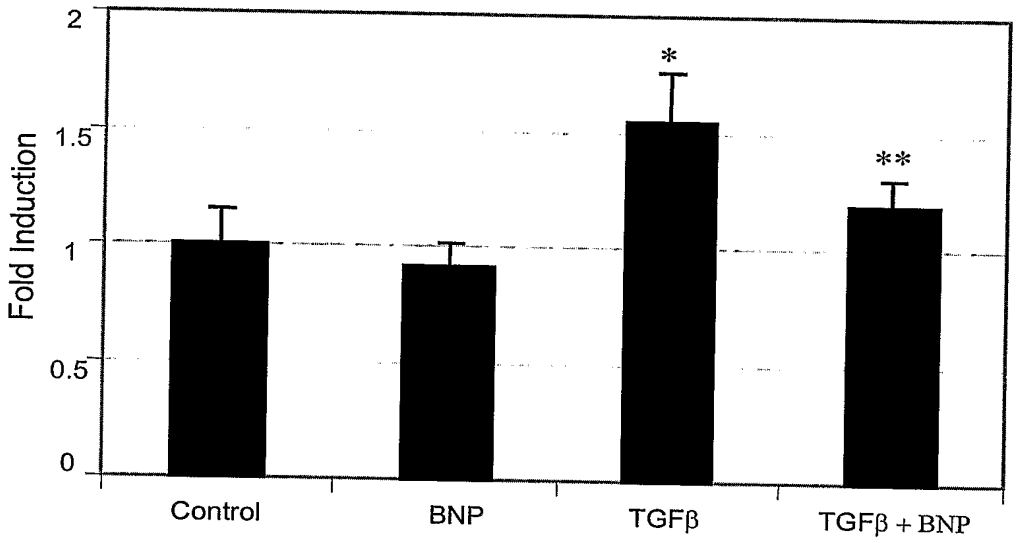


Figure 10

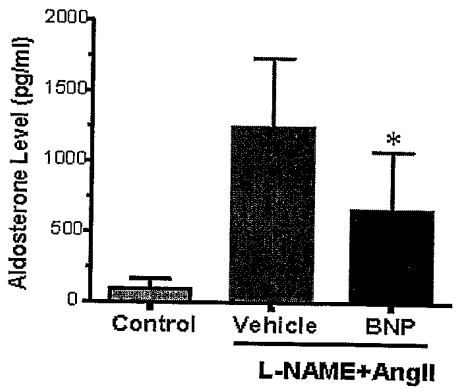

2000
1500
1000
500
0
Aldosterone Level (pg/ml)
*
Control
Vehicle
BNP
L-NAME+AngII

Figure 11

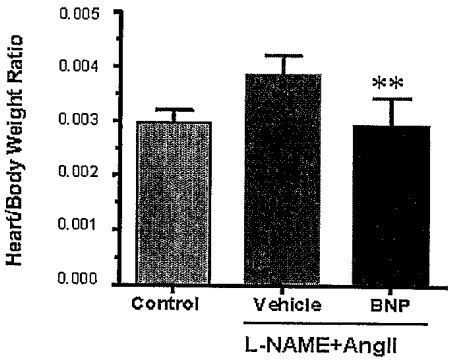

Heart/Body Weight Ratio
0.005
0.004
0.003
0.002
0.001
0.000
**
Control
Vehicle
BNP
L-NAME+AngII

Figure 12

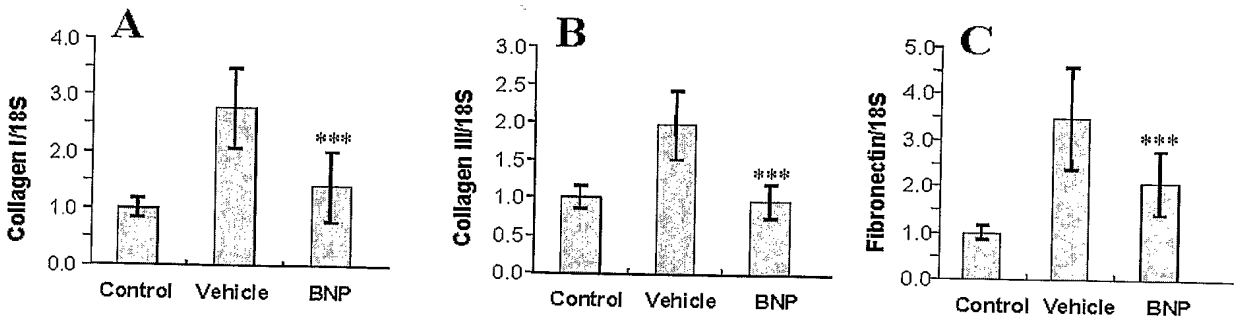
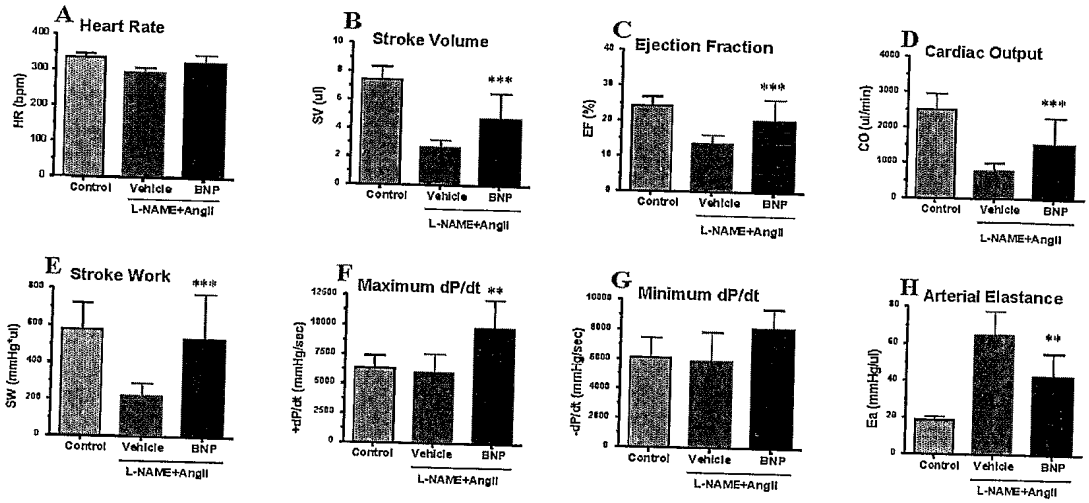

A
Collagen I/18S
4.0
3.0
2.0
1.0
0.0
***
Control
Vehicle
BNP
B
Collagen III/18S
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
Control
Vehicle
BNP
C
Fibronectin/18S
5.0
4.0
3.0
2.0
1.0
0.0
***
Control
Vehicle
BNP

Figure 13


A
Heart Rate
HR (bpm)
Control
Vehicle
BNP
L-NAME+AngII
B
Stroke Volume
SV (ul)
***
C
Ejection Fraction
EF (%)
D
Cardiac Output
CO (ul/min)
E
Stroke Work
SW (mmHg*ul)
F
Maximum dP/dt
+dP/dt (mmHg/sec)
G
Minimum dP/dt
-dP/dt (mmHg/sec)
H
Arterial Elastance
Ea (mmHg/ul)
**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US05/01480

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 38/00
US CL : 514/12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN EAST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Hayashi et al. Intravenous Atrial Natriuretic Peptide Prevents Left Ventricular Remodeling in Patients With Anterior Acute Myocardial Infarction. Journal of the American College of Cardiology, Feb. 2000. Vol. 35, No. 2 suppl. A, page 345A. See entire document.	1-2 --- 4
X	Fernándes et al. Cardiac remodeling in patients with systemic sclerosis with no signs or symptoms of heart failure: An endomyocardial biopsy study. J. Cardiac Failure, August 2003, Vol.9, No.4, abstract only, page 1. See last paragraph.	3
Y	Diez et al. Losartan-Dependent Regression of Myocardial Fibrosis Is Associated With Reduction of Left Ventricular Chamber Stiffness in Hypertensive Patients. Circulation, 2002. Vol. 105: pages 2512-2517.	5-8
X,P --- Y,P	Tsuneyoshi et al. Atrial Natriuretic Peptide Helps Prevent Late Remodeling After Left Ventricular Aneurysm Repair. Circulation. 2004: 110:II-174-II179 (abstract attached, pages 1-2). See entire document, e.g., page 2, last paragraph.	1-4

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search
16 March 2005 (16.03.2005)

Date of mailing of the international search report
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Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US05/01480

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	Kapoun et al. B-Type Natriuretic Peptide Exerts Broad Functional Opposition to Transforming Growth Factor-B in Primary Human Cardiac Fibroblasts. Fibroses, Myofibroblast Conversion, Proliferation, and Inflammation. Circulation Research, March 5, 2004. Vol. 94. No. 4, pages 453-461. See entire document, e.g., abstract and pages 459-460.	1-8